Cord Brakebusch
Taina Pihlajaniemi
Editors

Mouse as a Model Organism

From Animals to Cells

Springer

Mouse as a Model Organism

Cord Brakebusch · Taina Pihlajaniemi
Editors

Mouse as a Model Organism

From Animals to Cells

Springer

Editors
Cord Brakebusch
University of Copenhagen
Institute of Biomedicine, BRIC
Ole Maaloes Vej 5
DK-2200 Copenhagen
Denmark
cord.brakebusch@bric.ku.dk

Taina Pihlajaniemi
Oulu Center for Cell-Matrix
Research
FIN90014 Oulu
Finland
taina.pihlajaniemi@oulu.fi

ISBN 978-94-007-0749-8 e-ISBN 978-94-007-0750-4
DOI 10.1007/978-94-007-0750-4
Springer Dordrecht Heidelberg London New York

Library of Congress Control Number: 2011925376

Preface

The importance of mice as model organism has continuously increased throughout the last decades due to the widespread use of genetically modified mice. These mice significantly increased our understanding of the function of specific genes in a living mammalian organism during development and in disease. Ongoing efforts to create knockouts and conditional knockouts of all mouse genes by high-throughput gene targeting and phenotyping are expected to further boost the use of mice in biomedical research. In June 2009, a symposium on "Mouse as a Model Organism – From Animals to Cells" was held in Rovaniemi, Finland, trying to give an overview about recent developments and future directions in the filed. This conference, organized by the "Nordish Infrastructure for Mouse Models" (www.norimm.org) and supported by NordForsk, brought together distinguished scientists from all over the world to discuss these topics together with students from many places in Europe in the immediate vicinity of the polar circle.

Great research, the special atmosphere of Finnish Lapland in late spring with endless days and midnight sun, and not the least a meeting with Santa Claus himself in the nearby Santa Claus Village made the symposium a very unique experience. Out of that spirit and at the last day of the conference, the idea was born to combine review articles on different topics presented at that conference in a book. The response was very positive and the result can be seen on the following pages.

The first four chapters cover general aspects of generation and phenotyping of genetically modified mice, including the use of genomic insulators in transgenic constructs, the running of a "Mouse Clinic" for high-throughput phenotyping, the effects of genetic background and environment on the phenotype of mutant mice, and the requirements for a phenotyping database. The next chapters will then illustrate the use of mice as disease models and as a source for primary cells with cancer research as an example. This includes an overview about cancer models in mice and ex vitro and in vivo models for angiogenesis followed by a review on cancer associated fibroblasts and in vitro invasion assays. Finally, mouse models for investigating systemic cancer effects on indolent tumors will be described.

We hope that this book gives a good introduction into current possibilities in using mouse models to understand the molecular pathways underlying human diseases, and that it gives an outlook to the results to be expected from the high-throughput phenotyping of mouse mutants within the next years.

Copenhagen, Denmark — Cord Brakebusch
Oulu, Finland — Taina Pihlajaniemi

Contents

Contributors

Jerzy Adamski Institute of Experimental Genetics, Genome Analysis Center, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, adamski@helmholtz-muenchen.de

Thure Adler Institute for Medical Microbiology, Immunology and Hygiene, Technische Universität München, Munich, Germany, thure.adler@helmholtz-muenchen.de

Luciana Afonso Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, luciana.afonso@helmholtz-muenchen.de

Juan Antonio Aguilar Pimentel Division of Environmental Dermatology and Allergy, Technische Universität München/Helmholtz Zentrum München, Munich, Germany; Department of Dermatology and Allergy Biederstein, Technische Universität München, Munich, Germany, aguilar@helmholtz-muenchen.de

Lore Becker Department of Neurology, Friedrich-Baur-Institut, Ludwig-Maximilians-Universität München, Munich, Germany, lore.becker@helmholtz-muenchen.de

Johannes Beckers Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany; Lehrstuhl für Experimentelle Genetik, Technische Universität München, Freising-Weihenstephan, Germany, beckers@helmholtz-muenchen.de

Raffi Bekeredjian Division of Cardiology, Department of Medicine III, University of Heidelberg, Heidelberg, Germany, Raffi.Bekeredjian@med.uni-heidelberg.de

Sarah Berndt Groupe Interdisciplinaire de Génoprotéomique Appliquée-Cancer (GIGA-Cancer), Laboratory of Tumor and Developmental Biology, University of Liege, B-4000 Liège, Belgium, sarah.berndt@parisdescartes.fr

Marc Bracke Laboratory of Experimental Cancer Research, Department of Radiation Oncology and Experimental Cancer Research, Ghent University Hospital, 9000 Gent, Belgium, marc1.bracke@hotmail.com

Cory Brayton Molecular and Comparative Pathobiology, School of Medicine, Johns Hopkins University, Baltimore, MD, USA, cbrayton@jhmi.edu

Dirk H. Busch Institute for Medical Microbiology, Immunology and Hygiene, Technische Universität München, Munich, Germany, Dirk.Busch@mikrobio.med.tum.de

Julia Calzada-Wack Institute of Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, calzada@helmholtz-muenchen.de

Christian Cohrs Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, christian.cohrs@helmholtz-muenchen.de

Astrid De Boeck Laboratory of Experimental Cancer Research, Department of Radiation Oncology and Experimental Cancer Research, Ghent University Hospital, 9000 Gent, Belgium, astrid.deboeck@ugent.be

Olivier De Wever Laboratory of Experimental Cancer Research, Department of Radiation Oncology and Experimental Cancer Research, Ghent University Hospital, 9000 Gent, Belgium, olivier.dewever@UGent.be

Mehdi El Hour Groupe Interdisciplinaire de Génoprotéomique Appliquée-Cancer (GIGA-Cancer), Laboratory of Tumor and Developmental Biology, University of Liege, B-4000 Liège, Belgium, mehdi.el.hour@gmail.com

Irene Esposito Institute of Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany; Institute of Pathology, Technische Universität München, Munich, Germany, irene.esposito@helmholtz-muenchen.de

Almudena Fernández Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología (CNB), Consejo Superior de Investigaciones Científicas (CSIC), 28049 Madrid, Spain; Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), ISCIII, Madrid, Spain, afernandez@cnb.csic.es

Helmut Fuchs Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, hfuchs@helmholtz-muenchen.de

Valérie Gailus-Durner Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, gailus@helmholtz-muenchen.de

Hilary Gates MRC Mammalian Genetics Unit, Harwell, UK, h.gates@har.mrc.ac.uk

Donald Gullberg Department of Biomedicine, University of Bergen, Bergen, Norway, donald.gullberg@biomed.uib.no

John M. Hancock MRC Mammalian Genetics Unit, Harwell, UK, j.hancock@har.mrc.ac.uk

Wolfgang Hans Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, wolfgang.hans@helmholtz-muenchen.de

Ritva Heljasvaara Oulu Center for Cell-Matrix Research, Biocenter Oulu, University of Oulu, FIN-90014, Oulu, Finland; Department of Medical Biochemistry and Molecular Biology, Institute of Biomedicine, University of Oulu, FIN-90014, Oulu, Finland, ritva.heljasvaara@oulu.fi

An Hendrix Department of Medical Oncology, Ghent University Hospital, 9000, Gent, Belgium, USA, an.hendrix@ugent.be

Heinz Höfler Institute of Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, hoefler@helmholtz-muenchen.de

Marion Horsch Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, horsch@helmholtz-muenchen.de

Martin Hrabě de Angelis Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany; Lehrstuhl für Experimentelle Genetik, Technische Universität München, Freising-Weihenstephan, Germany, hrabe@helmholtz-muenchen.de

Koen Jacobs Laboratory of Experimental Cancer Research, Department of Radiation Oncology and Experimental Cancer Research, Ghent University Hospital, 9000 Gent, Belgium, koen.jacobs@ugent.be

Melanie Kahle Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, melanie.kahle@helmholtz-muenchen.de

Hugo Katus Division of Cardiology, Department of Medicine III, University of Heidelberg, Heidelberg, Germany, sekretariat_katus@med.uni-heidelberg.de

Martin Klingenspor Molecular Nutritional Medicine, ZIEL Research Center for Nutrition and Food Sciences, Technische Universität München, Freising-Weihenstephan, Germany, martin.klingenspor@wzw.tum.de

Thomas Klopstock Department of Neurology, Friedrich-Baur-Institut, Ludwig-Maximilians-Universität München, Munich, Germany, thomas.klopstock@med.uni-muenchen.de

Christoph Lengger Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, lengger@helmholtz-muenchen.de

Tonia Ludwig Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, tonia.ludwig@helmholtz-muenchen.de

Holger Maier Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, holger.maier@helmholtz-muenchen.de

Anne Masset Groupe Interdisciplinaire de Génoprotéomique Appliquée-Cancer (GIGA-Cancer), Laboratory of Tumor and Developmental Biology, University of Liege, B-4000 Liège, Belgium, Anne.Masset@ulg.ac.be

Sandra S. McAllister Harvard Medical School, Boston, MA, USA; Hematology, Brigham and Women's Hospital, Boston, MA, USA, smcallister1@partners.org

Kateryna Micklich Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, kateryna.butuzova@helmholtz-muenchen.de

Gabriele Möller Institute of Experimental Genetics, Genome Analysis Center, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, gabriele.moeller@helmholtz-muenchen.de

Eduardo Moltó Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología (CNB), Consejo Superior de Investigaciones Científicas (CSIC), 28049 Madrid, Spain; Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), ISCIII, Madrid, Spain, emolto@cnb.csic.es

Lluís Montoliu Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología (CNB), Consejo Superior de Investigaciones Científicas (CSIC), 28049 Madrid, Spain; Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), ISCIII, Madrid, Spain; montoliu@cnb.csic.es

Beatrix Naton Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, beatrix.naton@helmholtz-muenchen.de

Frauke Neff Institute of Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, frauke.neff@helmholtz-muenchen.de

Susanne Neschen Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, susanne.neschen@helmholtz-muenchen.de

Agnès Noel Groupe Interdisciplinaire de Génoprotéomique Appliquée-Cancer (GIGA-Cancer), Laboratory of Tumor and Developmental Biology, University of Liege, B-4000 Liège, Belgium, agnes.noel@ulg.ac.be

Markus Ollert Division of Environmental Dermatology and Allergy, Technische Universität München/Helmholtz Zentrum München, Munich, Germany; Department of Dermatology and Allergy Biederstein, Technische Universität München, Munich, Germany, ollert@lrz.tu-muenchen.de

Taina Pihlajaniemi Oulu Center for Cell-Matrix Research, Biocenter Oulu, University of Oulu, FIN-90014, Oulu, Finland; Department of Medical Biochemistry and Molecular Biology, Institute of Biomedicine, University of Oulu, FIN-90014, Oulu, Finland, taina.pihlajaniemi@oulu.fi

Cornelia Prehn Institute of Experimental Genetics, Genome Analysis Center, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, cornelia.prehn@helmholtz-muenchen.de

Birgit Rathkolb Institute of Molecular Animal Breeding and Biotechnology, Gene Center, Ludwig-Maximilians-Universität München, Munich, Germany, birgit.rathkolb@helmholtz-muenchen.de

Rolf K. Reed Department of Biomedicine, University of Bergen, Bergen, Norway, rolf.reed@biomed.uib.no

Jan Rozman Molecular Nutritional Medicine, ZIEL Research Center for Nutrition and Food Sciences, Technische Universität München, Freising-Weihenstephan, Germany, jan.rozman@helmholtz-muenchen.de

Markus Scheerer Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, markus.scheerer@helmholtz-muenchen.de

Evelyn Schiller Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, evelyn.schiller@helmholtz-muenchen.de

Felix Schöfer Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, felix.schoefer@helmholtz-muenchen.de

Anja Schrewe Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, anja.schrewe@helmholtz-muenchen.de

Patricia da Silva-Buttkus Institute of Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, tbuttkus@aol.com

Nor Eddine Sounni Groupe Interdisciplinaire de Génoprotéomique Appliquée-Cancer (GIGA-Cancer), Laboratory of Tumor and Developmental Biology, University of Liege, B-4000 Liège, Belgium, nesounni@ulg.ac.be

Ralph Steinkamp Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, ralph.steinkamp@helmholtz-muenchen.de

Claudia Stöger Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, claudia.stoeger@helmholtz-muenchen.de

Frank Thiele Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, frank.thiele@helmholtz-muenchen.de

Monica Tost Institute of Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, Monica.Tost@tiho-hannover.de

Irina Treise Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, irina.treise@helmholtz-muenchen.de

Cristina Vicente-García Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología (CNB), Consejo Superior de Investigaciones Científicas (CSIC), 28049 Madrid, Spain; Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), ISCIII, Madrid, Spain, cristina.vicente@cnb.csic.es

Wendy Westbroek Medical Genetics Branch, National Human Genome Research Institute, National Institute of Health, Bethesda, MD 20892, USA, wwestbro@mail.nih.gov

Monja Willershäuser Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, monja.willershaeuser@helmholtz-muenchen.de

Eckhard Wolf Institute of Molecular Animal Breeding and Biotechnology, Gene Center, Ludwig-Maximilians-Universität München, Munich, Germany, ewolf@lmb.uni-muenchen.de

Ramona Zeh Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany, ramona.zeh@helmholtz-muenchen.de

Chapter 1
Genomic Insulators in Transgenic Animals

Eduardo Moltó, Cristina Vicente-García, Almudena Fernández, and Lluís Montoliu

Abstract Vertebrate genomes are functionally and structurally organised as gene expression domains. These domains contain all regulatory elements required for the gene (or genes) to be expressed correctly, and include those required to shield each domain, thereby blocking any non-desirable interaction from their neighbours. These elements are known as "boundaries" or "insulators" and their function is to insulate gene expression domains in genomes allowing the protected locus to be expressed according to internal regulatory elements, without suffering from the adverse effects of flanking loci and without transmitting the effect of the internal regulatory elements beyond the protected domain. Insulators can act as "enhancer blockers", preventing a distal enhancer from interacting with a proximal promoter, when placed in between, and/or as "barriers", preventing chromosomal position effects associated with the genomic location. In addition, insulators are known to contribute to the chromatin and nuclear structural organization. A variety of molecular mechanisms have been associated with boundary function, probably reflecting the diversity of functional elements that can efficiently insulate genomic sequences. Insulator elements can be used in biotechnological applications, as spacers, as boundaries, and be applied to any gene expression construct to be used in gene transfer experiments (i.e. transgenesis, gene therapy), thereby preventing the inappropriate expression patterns of constructs and shielding them from neighbouring sequences surrounding the place of insertion in the host genomes.

L. Montoliu (✉)
Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología (CNB), Consejo Superior de Investigaciones Científicas (CSIC), 28049 Madrid, Spain; Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), ISCIII, Madrid, Spain
e-mail: montoliu@cnb.csic.es

Contribution for the book edited by Cord Brakebusch on the talks presented at the NorIMM (Nordic Infrastructure for Mouse Models) Symposium, held in Rovaniemi (Finland), June 2–4, 2009.

C. Brakebusch, T. Pihlajaniemi (eds.), *Mouse as a Model Organism*,
DOI 10.1007/978-94-007-0750-4_1,

1.1 Introduction

The eukaryotic genome is organized into genes or clusters of genes, associated to non-coding sequences, where regulatory elements that are required for the correct expression of these genes are located (Dillon, 2006). Surprisingly, coding sequences constitute only around 5% of the mammalian genomes, whereas the rest correspond to intergenic sequences that are full of repetitive and *cis*-regulatory sequences. Among all these regulatory elements, a new class has been recently defined and studied, the so-called *boundaries* or *insulators*, which maintain the structural and functional identity of the expression domains (Fig. 1.1). Insulators prevent inappropriate interactions between regulatory elements from neighbouring expression domains, and can create effective barriers against the spreading of adjacent heterochromatin, thus protecting against heterochromatin-mediated silencing (Fig. 1.2; Bell, West & Felsenfeld, 2001; Wallace & Felsenfeld, 2007; Moltó et al. 2009).

Usually, genes are not organized in chromosomes according to their expression patterns. Therefore it is not uncommon to find neuronal-specific genes located

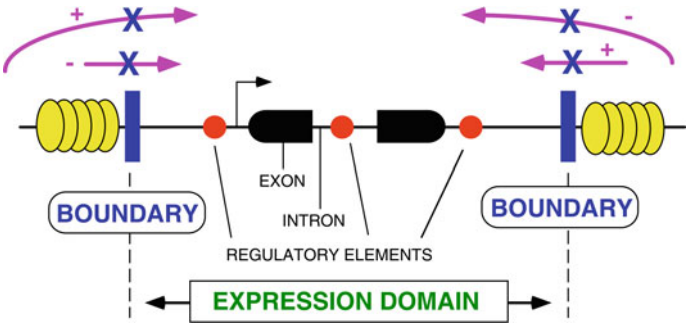

Fig. 1.1 Schematic representation of an expression domain from a vertebrate genome. Boundary elements and their protective function, shielding the locus from neighbouring interactions, are indicated

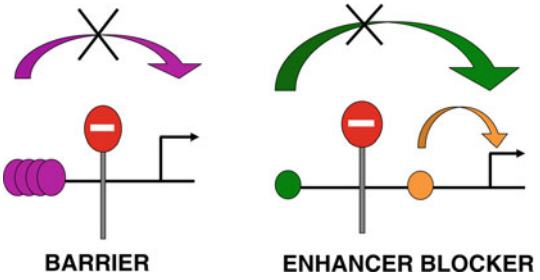

Fig. 1.2 The two main properties of genomic insulators: barrier function (*left*), preventing the negative effects of surrounding heterochromatin, and, enhancer-blocker function (*right*), interrupting the communication between distal enhancers and proximal promoters

next to muscle-specific genes or adjacent to ubiquitously-expressed loci. Such organization could generate arbitrary changes in gene expression at tissues and developmental stages where a given locus would normally not be expressed. In this scenario, boundaries would be the punctuation tools that genomes use to preserve the transcriptional integrity of loci, allowing the internal regulatory elements to operate without any potential interference from neighbouring loci and, at the same time, preventing the expansion of the effects mediated by the local regulatory elements towards these adjacent genes. Hence, boundaries contribute to define the gene function and the structural organization of the nucleus (Dillon & Sabbattini, 2000; de Laat & Grosveld, 2003; Capelson & Corces, 2004).

The identification and characterization of genomic insulators are not only interesting for our understanding about how vertebrate genomes are organized, but also allow to design new and improved gene transfer strategies, which would ensure the correct expression of heterologous DNA constructs at ectopic genomic locations. In this chapter, we will discuss the potential use of vertebrate insulators outside their endogenous genomic context, in gene transfer events, namely animal transgenesis, and new approaches for the functional validation of potential vertebrate boundaries.

1.2 Vertebrate Insulators in Animal Transgenesis

Genomic insulators have been considered as additional tools for designing improved gene transfer vectors. Their insulating properties at ectopic locations have been explored, to shield a given gene expression construct from neighbouring sequences and render it independent of its genomic location, thereby overcoming the chromosomal position effects commonly observed in standard gene transfer events that are triggered by the random integration of constructs in the host genome (Giraldo & Montoliu, 2001; Montoliu, 2002; Giraldo, Rival-Gervier, Houdebine & Montoliu, 2003a; Recillas-Targa, Valadez-Graham & Farrell, 2004; Montoliu, Roy, Regales & García-Díaz, 2009; Houdebine, 2010).

The use of genomic boundaries as spacers for insulating gene cassettes in complex multicistronic transgenic or knockout constructs, is one of the most promising areas where insulators are expected to contribute to animal transgenesis (Bessa et al., 2009; Moltó, Fernández & Montoliu, 2009). However, the addition of insulator elements to transgenes does not always correlate with a better performance or lack of undesirable effects (variegation, site-dependent expression, long-term stability, copy-number independency) in transgenic animals. The addition of boundary elements, such as the 5′HS4 insulator from the chicken beta-globin locus, might contribute to the success of the experiment but does not guarantee full level of expression in all transgenic animal lines produced, and, in particular, silencing of lines might not be prevented in all cases (Giraldo, Rival-Gervier, Houdebine & Montoliu, 2003a). High-level and ubiquitous expression of human CD55 and CD59 cDNAs in transgenic rabbits were initially observed using a vector containing the

chicken 5′HS4 insulator (Taboit-Dameron et al., 1999). Also, reduced variability was obtained in transgenic mice generated with a tyrosinase reporter construct with this insulator (Potts, Tucker, Wood & Martin, 2000). Improved outcome of its use in transgenic vectors was later reported (Jin, McKeehan & Wang, 2003; Hsiao et al., 2004; Guglielmi et al., 2005; Moriyama et al., 2007), although frequently lacking the required detailed analyses to adequately assess the potential benefits of the 5′HS4 insulator addition. Copy-number dependent, position-independent expression and lack of variegation were reported in transgenic mice generated with the erythrocyte-specific AE1 gene upon the addition of the 5′HS4 insulator (Frazar et al., 2003), but the same boundary did not prevent variegation or ectopic expression in independent studies (Rival-Gervier et al., 2003a). Furthermore, even though the addition of 5′HS4 produced an increased proportion of high-expressing lines, it did not sustain copy-number dependent expression in several transgenic experiments in animals (Boëda, Weil & Petit, 2001) or cells (Truffinet, Guglielmi, Cogné, & Denizot, 2005). Several independent reports have already indicated that the use of the paradigmatic chicken 5′HS4 element does not always produce the expected benefits in transgenic animals (Pantano et al., 2003; Klopstock, Levy, Olam, Galun & Goldenberg, 2007). The effects of the 5′HS4 element on the mouse X-chromosome indicated that this chicken insulator is able to block the repression of an X-linked transgene during development, when placed on the X-active chromosome, but cannot overcome the silencing and hypermethylation of the transgene on the X-inactive chromosome (Ciavatta, Kalantry, Magnuson & Smithies, 2006).

Protection against position effects was also observed in transgenic mice using the 5′HS5 insulator from the human beta-globin locus, analogous to the chicken 5′HS4 element (Li, Zhang, Han, Rohde & Stamatoyannopoulos, 2002), and the αEHS-1.4 insulator from the chicken alpha-globin locus (Recillas-Targa and Montoliu, unpublished results). Taken together, all these data suggest that the combinations of promoter, reporter/gene and insulator sequences explored so far might not be optimal. To date, no combination of boundary elements has resulted in reproducible and robust patterns of transgene expression, universally, in all different genomic environments analysed. However, in relative terms, the addition of insulators to standard gene transfer constructs appears to increase the probability of transgenic expression, as compared to that of unprotected constructs. Therefore, the inclusion of insulator elements in transgenic constructs should be considered beneficial. With the advent of new insulator elements being identified and characterized, additional transgenic experiments could be performed where constructs could be shielded by different insulator elements at either end, in order to test whether the combination of two different insulator elements increases the efficiency and shielding capacity of transgene expression. In this regard, in our laboratory, we have used three loci to characterize boundary elements in mammals: the mouse tyrosinase gene (*Tyr*), the mouse whey-acidic protein (*Wap*) gene and the mouse growth hormone gene (*Gh*).

1.3 Transgenes and Insulator Elements From *Tyr*, *Wap* and *Gh* Mouse Loci

These three loci share a tissue-specific expression and a tighter regulation during development. Mouse tyrosinase is expressed from embryonic day +10.5 in retinal pigment epithelial (RPE) cells and from day +16.5 in melanocytes of the skin, hair follicle, the inner ear and the eye (iris, ciliary body, and choroid) (Beermann, Schmid & Schütz, 1992; Giménez et al. 2003). *Wap* is expressed during the last week of pregnancy and the first two weeks of lactation in the alveolar epithelial cells of the mammary gland (Burdon, Sankaran, Wall, Spencer & Hennighausen, 1991), whereas the mouse growth hormone is expressed from embryonic day +17.5 in the somatotrope cells of the anterior hypophysis (Slabaugh, Lieberman, Rutledge & Gorski, 1982).

Insulators are normally defined functionally according to the outcome of one or two experimental tests where their enhancer-blocking and barrier properties are assessed. The boundaries found in these three loci are composed by structurally different elements, as explained below.

The tyrosinase gene encodes the fundamental enzyme in melanin biogenesis and thus, the absence or malfunction of this gene results in oculocutaneous albinism type 1 (OCA1), a developmental disorder having an autosomal recessive mode of inheritance (Oetting, Fryer, Shriram & King, 2003; Ray, Chaki & Sengupta, 2007). A Locus Control Region (LCR) in the mouse tyrosinase gene was identified by transgenesis using yeast artificial chromosomes (YACs) that contained the mouse tyrosinase gene together with a sequence situated 15 kb upstream from the transcription start site (Montoliu, Umland & Schütz, 1996). Transgenic animals were able to completely rescue the wild type phenotype from albino recipient animals. However, upon removal of this upstream sequence, there was no rescue of the wild-type phenotype, resulting in a weak pigmentation and variable patterns of tyrosinase expression (Montoliu, Umland & Schütz, 1996; Giménez, Giraldo, Jeffery & Montoliu, 2001). Also, the extreme dilution mottled mutant mice ($Tyr^{c\text{-}em}$), arising from a chinchilla-mottled mutant stock ($Tyr^{c\text{-}m}$) and with a rearrangement of 5′-upstream regulatory sequences including the LCR of the *Tyr* gene, displayed a variegated pigmentation pattern in coat and eyes, in agreement with the LCR translocation, but also showed a generalized hypopigmented phenotype not seen in $Tyr^{c\text{-}m}$ mice (Lavado, Olivares, García-Borrón & Montoliu, 2005).

Later on, a boundary element was detected at the LCR of the mouse tyrosinase locus through in vivo functional analysis in transgenic flies and mice, and in vitro by enhancer-blocking assays (Giraldo et al., 2003b). This insulator appears to prevent the negative influence of neighbouring condensed chromatin on the LCR (Montoliu, Umland & Schütz, 1996), and is a fundamental regulatory element that appears to be conserved in the homologous human locus (Regales, Giraldo, García-Díaz, Lavado & Montoliu, 2003). The condensed chromatin is associated with a

hypermethylated LINE1 element, found upstream of the insulator element (Giraldo et al., 2003b). In the tyrosinase LCR, the boundary elements are situated specifically in the identified AB and G-rich boxes and in the G-rich where CTCF binding activities have been found (Montoliu L, unpublished results). Inactivation of the A and B elements in YACs in transgenic mice showed that both elements are important for the correct expression of tyrosinase, because, in their absence, the wild phenotype could not be rescued, and, instead, variegated tyrosinase-expression patterns were found (Moreira et al., 2004). Finally, ectopic boundary function has been demonstrated, ectopically, in zebrafish transgenic constructs carrying an artificial fusion of the AB and G boxes, called the GAB element (Bessa et al., 2009).

The major whey protein in mouse is a cysteine-rich protein called whey acidic protein (WAP) by Piletz et al. (1981). Two insulators with powerful enhancer-blocking activities have been found at the murine gene of WAP on chromosome 11, flanking the locus and associated with scaffold/matrix-attachment regions (S/MARs) (Millot, Montoliu, Fontaine, Mata & Devinoy, 2003; Montazer-Torbati et al., 2008). The role of these boundary elements, not directly associated to CTCF, would be to insulate this mammary-gland specific gene from two surrounding ubiquitously expressed genes, *Tbrg4*/*Cpr2* and *Ramp3*, as initially detected in other mammalian species (Rival-Gervier et al., 2003b).

The *Wap* gene was one of the first loci initially identified for biotechnological applications in transgenic animals. A high-level production of human growth hormone in the milk of transgenic mice was obtained using the upstream region of the rabbit *Wap* gene (Devinoy et al., 1994). Also, the pig *Wap* locus has been further studied and larger genomic pieces analysed in transgenic mice, including a 145 kb BAC transgene and its subsequent deletion derivatives, demonstrating that the presence of these additional surrounding genomic sequences, including evolutionary conserved regulatory elements, were sufficient to sustain high levels of transgene expression (Rival-Gervier, Viglietta, Maeder, Attal & Houdebine, 2002; Saidi et al., 2007). Actually, comparative analysis between rabbit and mouse genomes allowed the identification of additional Stat5-trancriptional factor binding sites and regulatory elements found outside the DNA sequences traditionally used in previous *Wap* transgenes for ensuring mammary gland expression (Millot, Montoliu, Fontaine, Mata & Devinoy, 2003; Devinoy et al., 2005).

The murine *Gh* genomic locus is found on mouse chromosome 11 and encompasses five genes with diverse expression patterns: *Scn4a* (skeletal muscle-specific), *Cd79b* (lymphocyte B-specific), *Gh*, *Tcam-1* (testicle-specific), and *Baf60b* (a component of a chromatin remodelling complex) (Lunyak et al., 2007). Recently, a new insulating mechanism based in the transcription of a tRNA-derived SINE B2 retrotransposon was described for this locus. The SINE B2 element played an instrumental role organizing the entire locus and showed properties of an enhancer-blocker and a barrier. This small DNA element, with a 190-bp long consensus sequence, contains two promoters (RNA polymerase III and II) transcribing in opposite directions. Upon activation of the RNA polymerase II promoter, active chromatin modifications are triggered resulting in the corresponding opening at a precise developmental stage, and thus allowing the interaction with

pituitary-specific nuclear factors, such as Pit-1, responsible for the direct transcription of the *Gh* locus (Lunyak et al., 2007; Lunyak, 2008).

1.4 Perspectives

The examples of the *Tyr*, *Wap* and *Gh* genes demonstrate that CTCF is only one of the many mechanisms used by vertebrate cells to organize and plan for insulating activities, where they are needed, using the tools and sequence elements that are available in each case. Indeed, it has been accepted, confirmed by experimental data and the continuous description of new insulators, that several types of insulator exist, operating under different mechanisms of action, through different molecular strategies (West, Gaszner & Felsenfeld, 2002; Lunyak, 2008). There are no trustable methods for predicting true functional insulators from their genomic structure or the presence of binding sites to known proteins (e.g. CTCF), without carrying out the corresponding functional tests. In particular, a good number of CTCF sites, detected in genomic studies, do not appear to bind CTCF in reality, if they are individually assessed, through chromatin immunoprecipitation analyses, nor they behave as insulators (Kim et al., 2007). And, surprisingly, the emerging consensus sequences encompassing all possible CTCF sites that have been found in the genome do not fit with the known binding sequences to determined CTCF sites in particular genes, where this protein does bind and does transduce insulator activity (Ohlsson, Renkawitz & Lobanenkov, 2001). Therefore, further imaginative screenings are required to identify novel insulators in vertebrate genomes, coupled with efficient and rapid functional tests that would select potential boundary candidates, to be eventually tested in vivo, for our better understanding of insulator function and its associated gene transfer applications.

In this regard, we are developing a new approach that will enable the systematic identification of most if not all possible mammalian genomic boundaries. The prediction of potential boundaries will be based on their genomic localization, according to a number of situations where the presence of boundaries is expected to be relevant. These situations would include: between loci with different expression patterns, thereby preventing interferences between regulatory elements of different domains; insulating expression domains from neighbouring heterochromatin; or, near crucial regulatory elements that command complex expression domains with similar expression pattern. Once identified *in silico*, the potential boundaries will be functionally validated, first in vitro, by enhancer-blocking assays (Lunyak et al., 2007), and then in vivo, using GFP-reporter constructs analysed in transgenic zebrafish (Bessa et al., 2009). In some selected cases, boundary elements could be eventually analysed in transgenic mice, using the mini-tyrosinase reporter constructs and their ability to rescue pigmentation using albino mouse embryos (Beerman et al., 1991).

The systematic functional validation of putative insulator sequences should allow our progress towards the understanding of the intrinsic role of these regulatory elements and their future ectopic use in biotechnological applications related to gene transfer events.

References

Beermann, F., Ruppert, S., Hummler, E., & Schütz, G. (1991). Tyrosinase as a marker for transgenic mice. *Nucleic Acids Research, 19*, 958.

Beermann, F., Schmid, E., & Schütz, G. (1992). Expression of the mouse tyrosinase gene during embryonic development: Recapitulation of the temporal regulation in transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America, 89*, 2809–2813.

Bell, A. C., West, A. G., & Felsenfeld, G. (2001). Insulators and boundaries: Versatile regulatory elements in the eukaryotic genome. *Science, 291*, 447–450.

Bessa, J., Tena, J. J., de la Calle-Mustienes, E., Fernández-Miñán, A., Naranjo, S., Fernández, A., Montoliu, L., Akalin, A., Lenhard, B., Casares, F., & Gómez-Skarmeta, J. L. (2009). Zebrafish enhancer detection (ZED) vector: A new tool to facilitate transgenesis and the functional analysis of cis-regulatory regions in zebrafish. *Developmental Dynamics, 238*, 2409–2417.

Boëda, B., Weil, D., & Petit, C. (2001). A specific promoter of the sensory cells of the inner ear defined by transgenesis. *Human Molecular Genetics, 10*, 1581–1589.

Burdon, T., Sankaran, L., Wall, R. J., Spencer, M., & Hennighausen, L. (1991). Expression of a whey acidic protein transgene during mammary development. Evidence for different mechanisms of regulation during pregnancy and lactation. *Journal of Biological Chemistry, 266*, 6909–6914.

Capelson, M., & Corces, V. G. (2004). Boundary elements and nuclear organization. *Biology of the Cell, 96*, 617–629.

Ciavatta, D., Kalantry, S., Magnuson, T., & Smithies, O. (2006). A DNA insulator prevents repression of a targeted X-linked transgene but not its random or imprinted X inactivation. *Proceedings of the National Academy of Sciences of the United States of America, 103*, 9958–9963.

de Laat, W., & Grosveld, F. (2003). Spatial organization of gene expression: The active chromatin hub. *Chromosome Research, 11,* 447–459.

Devinoy, E., Montoliu, L., Baranyi, M., Thépot, D., Hiripi, L., Fontaine, M. L., Bodrogi, L., & Bosze, Z. (2005). Analysis of the efficiency of the rabbit whey acidic protein gene 5′ flanking region in controlling the expression of homologous and heterologous linked genes. *Journal of Dairy Science, 72*, 113–119.

Devinoy, E., Thépot, D., Stinnakre, M. G., Fontaine, M. L., Grabowski, H., Puissant, C., Pavirani, A., & Houdebine, L. M. (1994). High level production of human growth hormone in the milk of transgenic mice: The upstream region of the rabbit whey acidic protein (WAP) gene targets transgene expression to the mammary gland. *Transgenic Research, 3*, 79–89.

Dillon, N., & Sabbattini, P. (2000). Functional gene expression domains: Defining the functional unit of eukaryotic gene regulation. *Bioessays, 22*, 657–665.

Dillon, N. (2006). Gene regulation and large-scale chromatin organization in the nucleus. *Chromosome Research, 14*, 117–126.

Frazar, T. F., Weisbein, J. L., Anderson, S. M., Cline, A. P., Garrett, L. J., Felsenfeld, G., Gallagher, P. G., & Bodine, D. M. (2003). Variegated expression from the murine band 3 (AE1) promoter in transgenic mice is associated with mRNA transcript initiation at upstream start sites and can be suppressed by the addition of the chicken beta-globin 5′ HS4 insulator element. *Molecular Cell Biology, 23,* 4753–4763.

Giménez, E., Giraldo, P., Jeffery, G., & Montoliu, L. (2001). Variegated expression and delayed retinal pigmentation during development in transgenic mice with a deletion in the locus control region of the tyrosinase gene. *Genesis, 30*, 21–25.

Giménez, E., Lavado, A., Giraldo, P., & Montoliu, L. (2003). Tyrosinase gene expression is not detected in mouse brain outside the retinal pigment epithelium cells. *European Journal of Neuroscience, 18*, 2673–2676.

Giraldo, P., Martínez, A., Regales, L., Lavado, A., García-Díaz, A., Alonso, A., Busturia, A., & Montoliu, L. (2003b). Functional dissection of the mouse tyrosinase locus control region identifies a new putative boundary activity. *Nucleic Acids Research, 31*, 6290–6305.

Giraldo, P., & Montoliu, L. (2001). Size matters: Use of YACs, BACs and PACs in transgenic animals. *Transgenic Research, 10*, 83–103.

Giraldo, P., Rival-Gervier, S., Houdebine, L. M., & Montoliu, L. (2003a). The potential benefits of insulators on heterologous constructs in transgenic animals. *Transgenic Research, 12*, 751–755.

Guglielmi, L., Truffinet, V., Carrion, C., Le Bert, M., Cogné, N., Cogné, M., & Denizot, Y. (2005). The 5′HS4 insulator element is an efficient tool to analyse the transient expression of an Em mu-GFP vector in a transgenic mouse model. *Transgenic Research, 14*, 361–364.

Houdebine, L. M. (2010). Design of expression cassettes for the generation of transgenic animals (including insulators). *Methods in Molecular Biology, 597*, 55–69.

Hsiao, Y. C., Chang, H. H., Tsai, C. Y., Jong, Y. J., Horng, L. S., Lin, S. F., & Tsai, T. F. (2004). Coat color-tagged green mouse with EGFP expressed from the RNA polymerase II promoter. *Genesis, 39*, 122–129.

Jin, C., McKeehan, K., & Wang, F. (2003). Transgenic mouse with high Cre recombinase activity in all prostate lobes, seminal vesicle, and ductus deferens. *Prostate, 57*, 160–164.

Kim, T. H., Abdullaev, Z. K., Smith, A. D., Ching, K. A., Loukinov, D. I., Green, R. D., Zhang, M. Q., Lobanenkov, V. V., & Ren, B. (2007). Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome. *Cell, 128*, 1231–1245.

Klopstock, N., Levy, C., Olam, D., Galun, E., & Goldenberg, D. (2007). Testing transgenic regulatory elements through live mouse imaging. *FEBS Letters, 581*, 3986–3990.

Lavado, A., Olivares, C., García-Borrón, J. C., & Montoliu, L. (2005). Molecular basis of the extreme dilution mottled mouse mutation: A combination of coding and noncoding genomic alterations. *Journal of Biological Chemistry, 280*, 4817–4824.

Li, Q., Zhang, M., Han, H., Rohde, A., & Stamatoyannopoulos, G. (2002). Evidence that DNase I hypersensitive site 5 of the human beta-globin locus control region functions as a chromosomal insulator in transgenic mice. *Nucleic Acids Research, 30*, 2484–2491.

Lunyak, V. V., Prefontaine, G. G., Núñez, E., Cramer, T., Ju, B. G., Ohgi, K. A., Hutt, K., Roy, R., García-Díaz, A., Zhu, X., Yung, Y., Montoliu, L., Glass, C. K., & Rosenfeld, M. G. (2007). Developmentally regulated activation of a SINE B2 repeat as a domain boundary in organogenesis. *Science, 317*, 248–251.

Lunyak, V. V. (2008). Boundaries. Boundaries. . . Boundaries??? *Current Opinion in Cell Biology, 20*, 281–287.

Millot, B., Montoliu, L., Fontaine, M. L., Mata, T., & Devinoy, E. (2003). Hormone-induced modifications of the chromatin structure surrounding upstream regulatory regions conserved between the mouse and rabbit whey acidic protein genes. *Biochemical Journal, 372*, 41–52.

Moltó, E., Fernández, A., & Montoliu, L. (2009). Boundaries in vertebrate genomes: Different solutions to adequately insulate gene expression domains. *Briefings in functional genomics & proteomics, 8*, 283–296.

Montazer-Torbati, M. B., Hue-Beauvais, C., Droineau, S., Ballester, M., Coant, N., Aujean, E., Petitbarat, M., Rijnkels, M., & Devinoy, E. (2008). Epigenetic modifications and chromatin loop organization explain the different expression profiles of the Tbrg4, WAP and Ramp3 genes. *Experimental Cell Research, 314*, 975–987.

Montoliu, L., Roy, R., Regales, L., & García-Díaz, A. (2009). Design of vectors for transgene expression: The use of genomic comparative approaches. *Comparative Immunology, Microbiology and Infectious Diseases, 32*, 81–90.

Montoliu, L., Umland, T., & Schütz, G. (1996). A locus control region at –12 kb of the tyrosinase gene. *EMBO Journal*, *15*, 6026–6034.

Montoliu, L. (2002). Gene transfer strategies in animal transgenesis. *Cloning Stem Cells, 4*, 39–46.

Moreira, P. N., Giraldo, P., Cozar, P., Pozueta, J., Jiménez, A., Montoliu, L., & Gutiérrez-Adán, A. (2004). Efficient generation of transgenic mice with intact yeast artificial chromosomes by intracytoplasmic sperm injection. *Biology of Reproduction, 71*, 1943–1947.

Moriyama, A., Kii, I., Sunabori, T., Kurihara, S., Takayama, I., Shimazaki, M., Tanabe, H., Oginuma, M., Fukayama, M., Matsuzaki, Y., Saga, Y., & Kudo, A. (2007). GFP transgenic mice

reveal active canonical Wnt signal in neonatal brain and in adult liver and spleen. *Genesis, 45*, 90–100.

Oetting, W. S., Fryer, J. P., Shriram, S., & King, R. A. (2003). Oculocutaneous albinism type 1: The last 100 years. *Pigment Cell Research, 16*, 307–311.

Ohlsson, R., Renkawitz, R., & Lobanenkov, V. (2001). CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. *Trends in Genetics, 17*, 520–527.

Pantano, T., Rival-Gervier, S., Prince, S., Menck-Le Bourhis, C., Maeder, C., Viglietta, C., Houdebine, L. M., & Jolivet, G. (2003). In vitro and in vivo effects of a multimerized alphas 1-casein enhancer on whey acidic protein gene promoter activity. *Molecular Reproduction and Development, 65*, 262–268.

Piletz, J. E., Heinlen, M., & Ganschow, R. E. (1981). Biochemical characterization of a novel whey protein from murine milk. *Journal of Biological Chemistry, 256*, 11509–11516.

Potts, W., Tucker, D., Wood, H., & Martin, C. (2000). Chicken beta-globin 5′HS4 insulators function to reduce variability in transgenic founder mice. *Biochemical and Biophysical Research Communications, 273*, 1015–1018.

Ray, K., Chaki, M., & Sengupta, M. (2007). Tyrosinase and ocular diseases: some novel thoughts on the molecular basis of oculocutaneous albinism type 1. *Progress in Retinal and Eye Research, 26*, 323–358.

Recillas-Targa, F., Valadez-Graham, V., & Farrell, C. M. (2004). Prospects and implications of using chromatin insulators in gene therapy and transgenesis. *Bioessays, 26*, 796–807.

Regales, L., Giraldo, P., García-Díaz, A., Lavado, A., & Montoliu, L. (2003). Identification and functional validation of a 5′ upstream regulatory sequence in the human tyrosinase gene homologous to the locus control region of the mouse tyrosinase gene. *Pigment Cell Research, 16*, 685–692.

Rival-Gervier, S., Pantano, T., Viglietta, C., Maeder, C., Prince, S., Attal, J., Jolivet, G., & Houdebine, L. M. (2003a). The insulator effect of the 5′HS4 region from the beta-globin chicken locus on the rabbit WAP gene promoter activity in transgenic mice. *Transgenic Research, 12*, 723–730.

Rival-Gervier, S., Thépot, D., Jolivet, G., & Houdebine, L. M. (2003b). Pig whey acidic protein gene is surrounded by two ubiquitously expressed genes. *Biochimica et Biophysica Acta, 1627*, 7–14.

Rival-Gervier, S., Viglietta, C., Maeder, C., Attal, J., & Houdebine, L. M. (2002). Position-independent and tissue-specific expression of porcine whey acidic protein gene from a bacterial artificial chromosome in transgenic mice. *Molecular Reproduction and Development, 63*, 161–167.

Saidi, S., Rival-Gervier, S., Daniel-Carlier, N., Thépot, D., Morgenthaler, C., Viglietta, C., Prince, S., Passet, B., Houdebine, L. M., & Jolivet, G. (2007). Distal control of the pig whey acidic protein (WAP) locus in transgenic mice. *Gene, 401*, 97–107.

Slabaugh, M. B., Lieberman, M. E., Rutledge, J. J., & Gorski, J. (1982). Ontogeny of growth hormone and prolactin gene expression in mice. *Endocrinology, 110*, 1489–1497.

Taboit-Dameron, F., Malassagne, B., Viglietta, C., Puissant, C., Leroux-Coyau, M., Chéreau, C., Attal, J., Weill, B., & Houdebine, L. M. (1999). Association of the 5′HS4 sequence of the chicken beta-globin locus control region with human EF1 alpha gene promoter induces ubiquitous and high expression of human CD55 and CD59 cDNAs in transgenic rabbits. *Transgenic Research, 8*, 223–235.

Truffinet, V., Guglielmi, L., Cogné, M., & Denizot, Y. (2005). The chicken beta-globin HS4 insulator is not a silver bullet to obtain copy-number dependent expression of transgenes in stable B cell transfectants. *Immunology Letters, 96*, 303–304.

Wallace, J. A, & Felsenfeld, G. (2007). We gather together: insulators and genome organization. *Current Opinion in Genetics & Development, 17*, 400–407.

West, A. G., Gaszner, M., & Felsenfeld, G. (2002). Insulators: Many functions, many mechanisms. *Genes & Development, 16*, 271–288.

Chapter 2
The German Mouse Clinic – Running an Open Access Platform

Valérie Gailus-Durner, Beatrix Naton, Thure Adler, Luciana Afonso, Juan Antonio Aguilar-Pimentel, Lore Becker, Julia Calzada-Wack, Christian Cohrs, Patricia da Silva-Buttkus, Wolfgang Hans, Marion Horsch, Melanie Kahle, Christoph Lengger, Tonia Ludwig, Holger Maier, Kateryna Micklich, Gabriele Möller, Frauke Neff, Susanne Neschen, Cornelia Prehn, Birgit Rathkolb, Jan Rozman, Evelyn Schiller, Anja Schrewe, Markus Scheerer, Felix Schöfer, Ralph Steinkamp, Claudia Stöger, Frank Thiele, Monica Tost, Irina Treise, Monja Willershäuser, Ramona Zeh, Jerzy Adamski, Raffi Bekeredjian, Johannes Beckers, Irene Esposito, Heinz Höfler, Hugo Katus, Martin Klingenspor, Thomas Klopstock, Markus Ollert, Eckhard Wolf, Dirk H. Busch, Helmut Fuchs, and Martin Hrabě de Angelis

Abstract Next challenges in functional annotation of mammalian genomes are yet of a much larger scope than previous genomics initiatives. Mouse mutant resources must be phenotyped systematically (one after the other) and systemically (assessing all organ systems). In addition, for the next generation of mouse models the "envirotypes", that humans are exposed to, need to be modeled. We established the German Mouse Clinic (GMC) as a mouse phenotyping platform for systemic, standardized phenotypic analysis and interpretation and open access for the scientific community on a collaborative basis. Next to phenotyping mouse models from national and international programs we provide the logistics to analyse mutant mouse lines from different origins (e.g., Knock-outs, Knock-ins, Gene-traps, RNAi Knock-downs, ENU mutagenesis, QTL studies) from collaboration partners throughout the world. Here we present an overview of how we organize a high-throughput screening of mouse lines from different laboratories and describe specific methods of selected screens in more detail.

V. Gailus-Durner (✉)
Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich/Neuherberg, Germany
e-mail: gailus@helmholtz-muenchen.de

C. Brakebusch, T. Pihlajaniemi (eds.), *Mouse as a Model Organism*,
DOI 10.1007/978-94-007-0750-4_2,

2.1 Introduction

Mouse models are essential tools for studying complex physiological networks in mammalian organisms and to determine mechanisms, dynamics, and exact disturbances leading to distinct diseases. Several techniques have been established over the past decades for the generation of mouse lines bearing defined genetic differences (Paigen, 2003a; b; Peters et al., 2007), constitutive and conditional gene knockout (Austin et al., 2004; Guan, Ye, Yang & Gao, 2010), and transgenic technology (Wu, Liu & Jaenisch, 1994; Enard et al., 2009), as well as genome-wide mutagenesis approaches such as ethylnitrosourea (ENU)-induced (Hrabé de Angelis et al., 2000, Nolan et al., 2000; Soewarto, Klaften & Rubio-Aliaga, 2009) and gene-trap-mediated methods (Hansen et al., 2003; 2008). The International Knockout Mouse consortium[1] (Collins, Finnell, Rossant & Wurst, 2007a; Collins, Rossant, Wurst & International Mouse Knockout Consortium, 2007b) aims to mutate all protein-coding genes in the mouse using a combination of gene trapping and gene targeting for the scientific community. Recently developed approaches include the use of transposons for random mutagenesis (Carlson et al., 2003) and RNA-induced gene silencing (through RNAi, Ying & Lin, 2009).

Many mutant mouse lines closely resemble clinical changes also found in human diseases (e.g., van Buerck et al., 2010; Rosemann et al., 2010; Aigner et al., 2007; 2008; Speakman, Hambly, Mitchell & Król, 2008). Those phenotypes are of particular interest for biomedical research, since they not only increase our understanding of pathomechanisms, they also provide the opportunity to test and develop new therapeutic strategies (e.g., Lisse et al., 2008; Pawlak et al., 2008; Altmaier et al., 2008). Since the number of available mouse mutants increases rapidly, comprehensive systemic phenotyping is the bottleneck to use the mouse mutant resource for biomedical research.

The mouse is a highly complex model system in which physiological as well as pathophysiological processes are strongly influenced by genetic and environmental factors. Together with the fact that mutations in mice usually affect many different molecular pathways, cell types, and organs, as it is for human syndromes, a detailed, systematic and comprehensive phenotypical analysis of these models is essential. Since specialized laboratories focus on their specific biological interest the acquired information is often limited to a specific research field, the available equipment and phenotypical tests. Thus, a comprehensive assessment and interpretation of all phenotypical data for a given mouse line is difficult to obtain, and important aspects of a particular mouse line are likely to be overlooked.

Therefore, we established an open-access platform for phenotypical analysis of mutant mouse lines, the "German Mouse Clinic (GMC)" (Gailus-Durner, Fuchs, & Becker, 2005, 2009; Fuchs et al., 2009). The concept of the GMC as an open

[1]Partners are the US-based Kockout Mouse Project (KOMP, http://www.knockoutmouse.org/) and Texas A&M Institute of Genomic Medicine (TIGM, http://www.tigm.org/), the European Conditional Mouse Mutagenesis (EUCOMM, http://www.eucomm.org/) and the North American Conditional Mouse Mutagenesis Project (NorCOMM, http://www.norcomm.org/) in Canada.

access platform for standardized, comprehensive phenotyping of mouse models on the basis of a scientific collaboration is still unique. The GMC is partner of large international phenotyping networks such as EUMODIC (The European Mouse Disease Clinic, www.eumodic.org/), Infrafrontier (www.infrafrontier.eu/), and IMPC (International Mouse Phenotyping Consortium; Brown, Chambon, Hrabé de Angelis & Eumorphia Consortium, 2005; Green et al., 2005; Mallon, Blake & Hancock, 2008).

2.2 Standardized Workflow of the Primary Screen at the German Mouse Clinic

We have developed a very complex logistics to import up to 3 mouse lines per week. Up to now, mutant mouse lines from 58 institutions from 16 different countries have been phenotyped. Specific requirements for cooperations are outlined in more detail on the website of the GMC (http://www.mouseclinic.de/). Mutant mouse lines entering the GMC are examined in a primary screen according to a workflow of consecutive phenotypic tests that evolved over the years (Fig. 2.1, modified after Gailus-Durner et al., 2005; Brown et al., 2005; Fuchs et al., 2009; Gailus-Durner et al., 2009). In the beginning of the GMC we analyzed a cohort of 60 mice

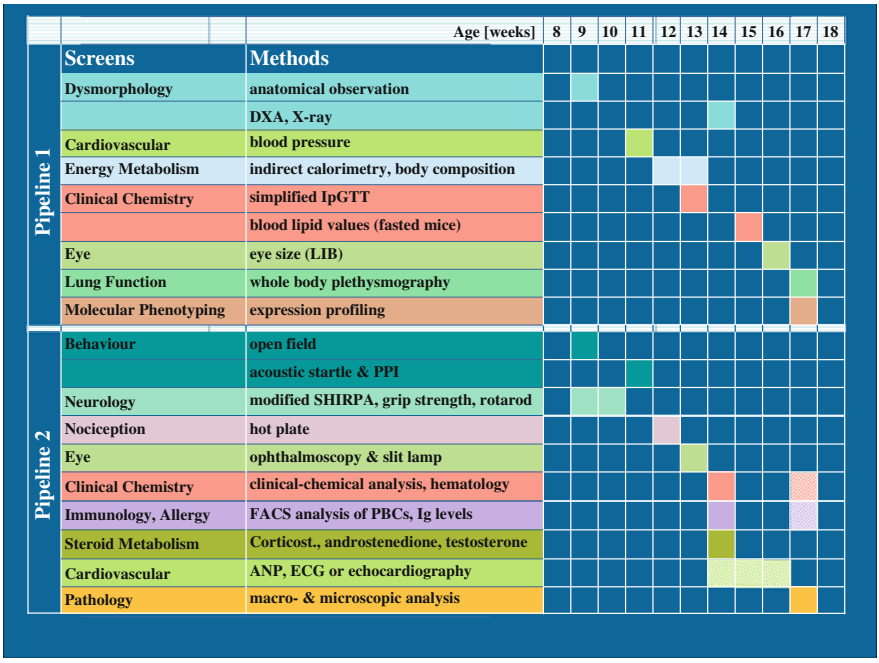

Fig. 2.1 Workflow of the primary screen

(30 mutant males and females together with the respective control littermates) in a single pipeline of tests. The duration of this analysis was around 10 weeks including a macroscopic and microscopic pathological analysis of every individual mouse.

Within the common effort of EUMODIC (The European Mouse Disease Clinic) to phenotype mouse mutants from programs generating mutant resources for all mouse genes (e.g., EUCOMM, the European Conditional Mouse Mutagenesis Programme, http://www.eucomm.org/) we have designed together with the EUMODIC partners a new workflow for primary phenotyping. To accommodate the different needs of the four mouse clinics ICS Strasbourg (http://www-ics.u-strasbg.fr/), MRC Harwell (http://www.har.mrc.ac.uk/), the Sanger Institute (http://www.sanger.ac.uk/) and the GMC with respect to equipment, mouse facilities and animal welfare we have developed a phenotypic analysis in two pipelines as depicted in Fig. 2.1. The mouse clinics have the first test batteries of the pipelines in common, the so called EMPReSS slim primary phenotyping screen (EMPReSS, European Mouse Phenotyping Resource for Standardized Screens, http://empress.har.mrc.ac.uk/). All EMPReSS slim protocols and additional validated phenotyping procedures are freely available from the EMPReSS website. At the GMC we perform additional GMC-specific tests at the end of the pipelines, namely the measurement of eye size and spontaneous breathing pattern, RNA expression profiling of selected organs, the measurement of the steroids testosterone and DHEA, echocardiography and the pathological analysis.

Since methods in phenotyping are developing rapidly, e.g., concerning automatization and data capture, the order and number of tests will again change in the future. Pathology, although time and cost intensive, gives essential information for a comprehensive functional annotation of the mouse genome. We learned that important information would be missed without thoroughly pathological examination like, e.g., the penetrance of a hydrocephalus in a mouse line (100% vs. obvious 25%, manuscript in prep.) or that the pathological findings already give an explanation for phenotypes detected in other screens (please see below). Other tests will be implemented if they can be performed in a high-throughput manner, give an important read-out and do not influence the outcome of subsequent measurements within the workflow. Changes in the pipeline will also evolve through further adjustments of a common phenotyping approach on the international level (e.g., within the International Mouse Phenotyping Consortium IMPC).

Performed tests are listed in Table 2.1. After the mice (a cohort of 80 female and male mice) arrive at the GMC, they are acclimatized to the new environment for two weeks and divided into two groups to enter the GMC in two pipelines (Fig. 2.1).

The mice of **the first pipeline** are subjected to a morphological whole-body checkup in the Dysmorphology Screen. In the Cardiovascular Screen blood pressure is measured and in the next module energy metabolism is analyzed by indirect calorimetry. In the following week a simplified intraperitoneal glucose tolerance Test is performed by the Clinical Chemical Screen. As next step, the mice re-enter the Dysmorphology screen for X-ray and DXA analysis. After the determination of eye size parameters and clinical chemical parameters from overnight fasted mice,

Table 2.1 Primary screen at the GMC

Screens	Goal	Methods
Dysmorphology, bone and cartilage	Morphological analysis of body, skeleton, bone, and cartilage	Morphological observation, Bone densitometry, X-ray
Behavior	Locomotion and anxiety-related behavior Sensory motor gaiting	Open field Acoustic startle & PPI
Neurology	Assessment of muscle, spinocerebellar, sensory, and autonomic function	Modified SHIRPA protocol Grip strength Rotarod
Eye	Assessment of morphological alterations of the eye	Funduscopy Laser interference biometry Slit lamp biomicroscopy
Nociception	Detection of altered pain response	Hot plate assay
Energy Metabolism	Measurement of body weight, body temperature, activity, O_2 consumption, CO_2 production, respiratory exchange ratio	Indirect calorimetry
Clinical Chemistry and Hematology	Determination of clinical-chemical and hematological parameters in blood Glucose tolerance	Blood autoanalyzer, ABC-animal blood counter Simplified IpGTT
Steroid Metabolism	Analysis of steroid hormones in blood plasma: testosterone and DHEA	ELISA
Immunology	Analysis of peripheral blood samples for immunological parameters	Flow cytometry, Multiplex Bead Array
Allergy	Analysis of total plasma IgE	ELISA
Cardiovascular	Assessment of functional cardio-vascular parameters, ANP	Non-invasive tail-cuff blood pressure measurement, Surface limb ECG/Echo, Heart weight ELISA
Lung Function	Assessment of spontaneous breathing pattern	Whole body plethysmography (Buxco®)
Molecular Phenotyping	RNA expression profiling	DNA-chip technology
Pathology	Microscopic and macroscopic examination	Histology, Immunohistochemistry

spontaneous breathing pattern is analyzed. Organs from males are archived in liquid nitrogen for optional molecular phenotyping.

The **second pipeline** starts in the Behavior Screen. The initial screening of the experimentally naïve mice includes also neurological tests and lasts three weeks. One week later, the animals are tested in the Nociceptive Screen. Then, mice are tested for eye parameters using funduscopy and slit lamp. When the mice are 14 weeks old, blood is taken, and samples are distributed to the blood-based screens Clinical Chemistry and Hematology, Immunology, Cardiovascular, and Allergy. Determination of Nt proANP level in plasma samples, electrocardiography or

echocardiography are performed on request. Three weeks after testing of the first blood sample, a second sample is taken to confirm the findings and analyze levels of the steroids testosterone and DHEA. After completion of the primary screen all animals of the second pipeline are analyzed macro- and microscopically in the Pathology.

Large scale mouse phenotyping requires close interaction of experts from various fields, including mouse physiology and genetics, physicians, engineers, computer scientists and bioinformaticians to design and analyze the specific phenotyping methods. Analytical methods, tests and set-ups had and have to be adapted from men to mice (e.g., analysis of mouse plasma in a blood auto analyzer also used in human hospitals) and technical equipment and instruments must be designed for animals as small as mice (X-ray, micro-CT). The methods are constantly refined and new technologies are tested for their use in a high-throughput screen. Here we show different aspects of the phenotypic analysis in a selection of screens at the GMC, giving detailed information of a specific method or equipment, different approaches for high-throughput analysis and aspects that have to be considered when analyzing the data.

2.3 Workflow and Data Management at the GMC

With its primary phenotyping protocol, the GMC offers a standardized, systemic analysis of mutant mouse lines and littermate controls. In the primary screen 14 different modules or research areas contribute to the phenotyping workflow by analyzing mice or blood samples in a certain week following an elaborated schedule of phenotyping procedures.

The GMC receives phenotyping requests from institutions worldwide leading to an increasing number of collaboration partners who would like to send their mice at the preferred time. Thus managing the logistical aspects of scheduling numerous phenotyping projects performed in parallel is critical for keeping the utilization capacity at a high rate. In addition, long-lasting in-depth analyses in secondary and tertiary screen phenotyping projects also have to be planned.

The majority of mutant lines phenotyped in the GMC were raised in animal facilities of our external collaboration partners. Before these mice enter the first phenotyping procedures, our partners have to process a list of tasks, including signing a collaboration agreement, submitting recent health reports of the mice, sending a summary of what is already known about the mutant mouse line, etc. In the GMC core facility, a team of 6 scientists and technicians is involved in keeping close contact to our collaboration partners, making sure that information provided is processed quickly and reminding them of the deadlines. The most critical deadline is the day when the final matings are started to generate the offspring for phenotyping to ensure that the mice are examined in a standardized and narrow age range. For the majority of phenotyping requests our list of tasks and processes follows a similar schedule. To make sure that all tasks are performed in time and all team members

are informed about the status of each task of each project the bioinformatics group of the GMC has developed a database-driven software tool that supports the team in terms of task management. Required user input is minimal, the team members only need to report the results of their tasks. Overview and search pages facilitate finding the next tasks. E-mail reminders are sent out a few days before a deadline is reached to make sure that deadlines are met. A phenotyping project is started by accepting a phenotyping request which has been sent by filling in the request form on the GMC web site (http://www.mouseclinic.de/CoordDBRequest/jsp/index.jsp). With this form we obtain the essential information from our collaboration partners, the properties of the mutant mouse line and its generation, former publications, intellectual property rights, legal and administrative issues as well as the extent of the phenotyping project. When all conditions are fulfilled, a phenotyping slot is arranged with the collaboration partner.

When the mice arrive at the GMC, a unique mouse identifier is assigned in MausDB (download MausDB for free from http://www.helmholtz-muenchen.de/ieg/; Maier et al., 2008b), the laboratory information management system of the GMC. Afterwards, the mice are allocated to one of the eight groups according to their sex, genotype and the phenotyping pipeline they are going to pass through. Every individual mouse bound to be phenotyped in the GMC is scheduled to the procedures. For the mice of the same line analyzed in the same week automatically a work list is generated, which is the binding basis for daily work of the phenotypers. Work lists contribute to assuring that all mice of a group had the same pretreatments so that the results are achieved under the same conditions and hence are comparable.

Moving towards high-throughput phenotyping, we learned that a standardized mouse phenotyping protocol and workflow has to be accompanied in the same manner by a standardized data workflow. In the GMC, the phenotyping data workflow is defined by the following steps: data capture, data validation, data management (storage, backup) and data reporting (analysis, visualization and distribution).

Data capture is a critical step in this pipeline, as conversion of analogue information from the primary observation or measurement by a device into digital form takes place here. Wherever possible, data capture should be automated in order to avoid error-prone and time-consuming human interaction steps. Unfortunately, according to our experience many devices do not have well-defined and well-documented digital interfaces. It seems as if these devices have rather been designed as stand-alone systems than having the option of integrating them into a complex data workflow and informatics system in mind. Thus developing data capture interfaces for all phenotyping tests can be very complex and laborious. Data capture may involve several intermediate steps, such as small databases of the analyzing device, text or spreadsheet files. Because of the individual structure and format of the result data obtained for every kind of procedure, establishing appropriate mechanisms to further process the result data with minimal user input is essential. For some procedures, especially when visual examinations of the mice are performed, it may be advantageous for downstream analysis to reduce the diversity of the parameter values by using controlled vocabulary or ontologies.

Data capture has to be followed by data validation in order to avoid incorrect data to be further processed. Erroneous data can be generated at every single intermediate step during data capture or even before, e.g. due to wrong device settings, wrong composition of reagents etc. Again, data validation should be automated by rule-based systems. However, as rule-based systems can only detect errors they have been trained for, validation is necessary as final step including critical revision by a human expert.

Data management includes storage and backup of data in order to ensure data integrity and prevent data loss. In the GMC, data is stored in a central transactional database. Full as well as incremental backups are automatically performed in a tight interval schedule. In addition, data integrity is checked by appropriate scripts on a regular basis.

2.4 Bone and Cartilage Phenotyping in Mice

The skeleton comprises around 14% of the total body weight, and thus it is the third largest organ system in the human body, whereby adult humans have 206 individual bones. There are many disorders of the skeleton. One of the most common is osteoporosis characterized by low bone mass and micro-architectural deterioration of bone tissue which leads to an increased risk of fracture. Studies of naturally occurring and genetically engineered mutant mice have led to a dramatic increase in the understanding of bone biology (Karsenty & Wagner, 2002). In many cases they have been essential for the discovery and understanding of bone related human diseases like osteoporosis (Ralston & de Crombrugghe, 2006; McLean & Olsen, 2001; Rosen, Beamer & Donahue, 2001), osteogenesis imperfecta (Rauch & Glorieux, 2004; Chipman et al., 1993), osteoarthritis (Abe, Fuchs, Lisse, Hans & Hrabě de Angelis, 2006), and scoliosis (Giampietro et al., 2003) or limb defects (Mariani & Martin, 2003). In contrast to human studies, standardization and regulation of environment, life style, dietary factors, and physical activity, is comparatively easy in the mouse.

Methods used to identify skeletal phenotypes in genetically altered mice were adapted from human diagnostics like X-ray, dual energy X-ray absorption (DXA), peripheral quantitative computed tomography (pQCT) and micro-computed tomography (μCT). For a first-line phenotyping, a broad spectrum of parameters involving bone development, homeostasis and metabolism should be covered. A morphological observation of the mouse is the easiest way to obtain fast and reliable information about skeletal malformations (Fuchs, Schughart, Wolf, Balling & Hrabě de Angelis, 2000). Subsequently DXA and X-ray imaging should be performed since both techniques enable a high throughput non-invasive first-line phenotyping for bone and cartilage abnormalities. DXA analysis is based on the different absorption of X-rays by different organ systems (Sorenson, Duke & Smith, 1989) and is one of the most commonly used techniques for the diagnostics of osteoporosis and bone

mineralization defects. The main advantage of DXA is its proven, reproducible technology in measuring bone and body composition parameters rapidly, precisely and accurately (Lochmüller et al., 2001, Baroncelli et al., 1998). It provides measures of bone mineral density (BMD), bone mineral content (BMC), bone-free lean tissue mass (LTM), and fat mass (FM). X-ray analysis can diagnose various conditions like joint dislocations, degenerative conditions, congenital or metabolic bone diseases.

Mutants that demonstrated altered parameters in the first-line screen should be analyzed in secondary tests in more detail. Such tests include pQCT, μCT, measurement of markers of bone metabolism and hormonal regulation, fracture/stress parameters, skeleton preparation and in vitro analysis of bone cells to unravel and describe potential cellular causes of an observed bone alteration. pQCT technology is more sensitive than DXA and enables real volumetric bone density measurements. It separates cortical and trabecular bone compartments and thus can monitor metabolic changes very quickly and precisely (Gasser 2003, Schmidt et al., 2003). For in vivo monitoring of bone density, mass and architecture, pQCT analysis is restricted to locations of the appendicular skeleton and tail vertebra.

With the application of this bone and cartilage phenotyping protocol we identified and characterized a new mouse model for osteogenesis imperfecta (OI) termed *Aga2* (abnormal gait 2) with a dominant mutation in the terminal C-propeptide domain of *Col1a1* generated using the N-ethyl-N-nitrosourea (ENU) mutagenesis strategy (Lisse et al., 2008). Mutant mice exhibited phenotypic alterations which resemble OI, including reduced bone mass, multiple fractures, and early lethality. We provided for the first time evidence for the involvement of ER stress-related apoptosis in bone tissue of mutated mice. Also we described the *Ali18* (abnormal limb 18) mutant mouse line, the first non-induced mouse model for psoriatic arthritis, dermatitis and osteoporosis (Abe et al., 2006, 2008). *Ali18* mice exhibited rubor and swelling of footpads in hindlimbs in adults. Histological analysis showed infiltration of mixed populations of inflammatory cells into bone marrow, peripheral joints, and skin in the affected areas of *Ali18* mice. DXA, pQCT, and μCT analysis revealed generalized osteoporosis-like phenotypes in mutant animals. Recently we detected an osteopenic phenotype in a new mouse model for uromodulin-associated kidney disease with alterations in urea handling, energy and bone metabolism (Kemter et al., 2009) which was probably a result of chronic hypercalciuria.

2.5 Neurological Screening

Neurological diseases are common human disorders with increasing impact in an aging society and animal models provide a useful tool for the investigation of mechanisms involved and putative therapeutic approaches (Hafezparast, 2002). A clinical neurological examination in humans is a series of small tests and observations with

focus on gesture, posture, coordination and motor functions as well as checking reflexes, cranial nerves and vegetative functions. Many parameters can be easily transferred but some adjustments have to be made to translate the tests from man to mouse. Mice will e.g. not deliberately follow instructions like walking a distinct line or pressing hands and, depending on the method used, different confounding factors have to be included. The primary screening for differences in mutant mice should give a spectrum of effects of a mutation and provide some hints for further, more in-depth analysis and validation of the results (Schneider et al., 2006). Analogous to the human examination, the modified SHIRPA protocol covers most of the above mentioned neurological parameters in a qualitative way, mainly distinguishing between normal and abnormal.

More quantitative data e.g., for evaluation of muscle function by grip force is obtained by using a grid attached to a force gauge thus allowing the measurement of 2- and 4-paw-force a mouse applies when slowly pulled away from the grid. Motor coordination and balance can be measured by putting the mice on a rotating rod and recording the latency to fall down (Brooks & Dunnett, 2009). Using an acceleration mode of the rotation the mice are permanently required to adjust their stepping with increasing level of difficulty. In addition, the reason for the trial end (falling, jumping or passive rotation) is recorded to make sure the measured time is an indicator of coordination and not e.g. a consequence of non-compliance. Performing several trials consecutively with a small inter-trial interval allows also for the evaluation of a given difference in ongoing trials: some animals might learn slower, others get easier exhausted.

For data analysis one has to keep in mind that there may be additional factors to consider: e.g. even small bone malformations might reduce grip force. Body weight can directly and also indirectly – as an indicator of general health or co-segregating with a non-neurological/muscular disease – influence several parameters and should therefore always be recorded and considered for the evaluation of the data. On the other hand, impaired motor coordination will probably interfere with behavioral analysis (Crawley, 2008).

For standardization of tests it is indispensable that the data are collected under the same conditions and deviations are at least recorded. Especially tests with awake animals performing tasks are prone to environment effects like noise and other disturbances. Different levels of arousal and activity in response to stress will influence several behavioral components of tests and might e.g. change compliance of the animals in the grip force testing or interfere with rotarod performance. Ideal conditions include recordings at the same daytime as well as concurrent measurement of proper controls of the same age, sex, and source.

All the data collected give a pattern for basic neurological functions alone and in combination with the results of other disciplines an overall picture of a mutant phenotype. Major dysfunctions will appear immediately, others will only be noted after careful analysis the complete mosaic. The framework of information from a comprehensive high-throughput screening offers the evaluation of data also with an unbiased view on yet undiscovered correlations and will help to further decipher human disease and perspectives.

2.6 Screening of Clinical Chemical and Hematological Parameters in Genetically Modified Mice: Considerations How to Assess a Mutant Phenotype

Measurement of clinical chemical and hematological parameters belongs to the screening program applied to identify mutant phenotypes of mice in almost all mouse phenotyping centers worldwide (Fuchs et al., 2009; Wakana et al., 2009; Solberg et al., 2006). Modern analyzers allow the measurement of a broad spectrum of clinical chemical and hematological values in a small blood sample of about 350–500 μl (Rathkolb et al., 2000; Hough et al., 2002; Gailus-Durner et al., 2009). Doing so, we can easily detect many kinds of pathological consequences of the mutation investigated. However, in most of the cases obvious pathological effects are missing and only mild or subtle genotype-related differences in clinical chemical and/or hematological parameters are detected and have to be carefully evaluated to find indications for the impact of the genetic modification on organ function and metabolism. To achieve optimal results concerning specificity and sensitivity of the tests, several aspects have to be considered in experimental design, sample preparation, data evaluation and interpretation, which will be discussed in this contribution.

To avoid false positive and false negative calls in data evaluation equal numbers of mutant and control mice of approximately the same age should be investigated in parallel. The age of the mice may vary within a two-week-range in young adults, while the age range can be bigger in older mice and should be smaller in growing mice. Wild-type littermates of the tested mutant mice grown under the same conditions are optimally used as controls. If these are not available, mice of the same genetic background, age and sex as the mutants investigated can be used that should be optimally housed in the same mouse facility. The group size chosen depends on the effects that are intended to be detected. We test at least seven mice per sex and genotype, but cohorts of 10 mice or more per group are preferred.

To get reliable results a sample collection method is chosen that minimizes the risk of hemolysis. In our lab retroorbital puncture of isoflurane anesthetized mice proved to be the best method to obtain samples of high quality. For clinical chemical analyses Li-heparin plasma is used, while a small amount of EDTA-blood is needed to analyze hematological parameters.

If it is planned to measure both, clinical chemical parameters which are sensitive to sample storage temperature like free fatty acids (NEFA) and glycerol, as well as parameters which are falsified by hemolysis like potassium, ASAT, LDH, iron and UIBC, it is recommended to use two separately collected samples. Alternatively the Li-heparin sample can be split into two portions in different tubes of which one (about 100 μl) is stored on ice directly after collection, while the other (200 μl) is stored at room temperature to avoid hemolysis. Plasma is separated from cells by centrifugation at 5,000 × g for 10 min. For most of the measurements it can be diluted 1:2 with deionized water to increase the volume available. However, since

bilirubin, NEFA and glycerol values are very low in healthy mice these parameters should be measured in undiluted samples.

To get an overview of the results obtained we first look for genotype and sex effects as well as genotype x sex interaction effects in each parameter by applying an ANOVA procedure on the data, followed by a Welch-test on male and female data separately in case that a sex or genotype x sex interaction effect was detected. If the measured values are not normally distributed, a non parametric test (Wilcoxon rank-sum test) is used to detect genotype-related differences. If there are strong genotype-related differences detected, meaning that the mutant values are situated outside the physiological range of the respective background strain, it is usually easy to identify the most likely cause. However, more often we see small but significant genotype-related differences in several parameters with most of the absolute values of both, mutant and control mice, being situated within the data range found in wild-type mice of the background strain. In these cases, it is much more difficult to identify possible causes for the differences seen and to judge on the relevance of the findings. For this purpose it is useful to look for changes in certain groups of parameters that are physiologically associated with each other, and for differences seen in other screens that can support assumptions on possible causes. The following examples are based on our experiences (published and unpublished data) and demonstrate the complexity of clinical data.

Differences in the following parameters can indicate effects on kidney function: urea, creatinine, uric acid, total protein, albumin, α-amylase, alkaline phosphatase (ALP), calcium, magnesium, potassium, inorg. phosphorus, sodium, chloride and cholesterol.

In general disturbed kidney function is associated with increased plasma urea values, which made this test an effective screening parameter to identify ENU-induced mouse models of renal diseases (Aigner et al., 2007). In cases of tubular dysfunction, this can be associated with changes in plasma electrolyte levels and uric acid concentrations. Due to changes in calcium-phosphate balance, kidney dysfunction is sometimes associated with effects on bone metabolism indicated by increased ALP activities and changes in bone mineral density (DXA). In cases of proteinuria effects on plasma proteins, mainly albumin and α-amylase can be expected, while cholesterol levels increase in this case. In both cases we often see additional effects on energy metabolism.

As another example possible reasons for changes in the peripheral red blood cell count are discussed. The red blood picture is very uniform in inbred mice. Therefore quite small changes can be reliably detected in mutant mice. Changes in red blood cell size and number may occur due to several reasons:

Reduced red blood cell counts (RBCs) can result from reduced production, bleeding or intravascular hemolysis. Erythropenia associated with increased mean corpuscular volume (MCV) usually indicates effects on stem cell dividing frequency, which can be caused by vitamin deficiencies or direct effects on cell cycle, e.g., by ribosomal dysfunction. Therefore hints on possible causes can be found in findings of the metabolism screen (reduced food intake), dysmorphology screen (diarrhea) or pathology screen (changes in stomach or gut morphology). However, a

slightly increased MCV can also be a result of an increased reticulocyte number due to blood cell loss. Intravasal hemolysis would be associated with increased potassium and bilirubin values as well as lactate dehydrogenase (LDH) and aspartate aminotransferase (ASAT) activities, while impaired blood clotting would be associated with subcutaneous and/or intestinal bleedings which can be diagnosed by the pathology screen. Low RBCs without changes in cell size can be due to reduced erythropoietin levels and are therefore found associated with renal diseases.

Microcytosis (reduced MCV) occurs in iron metabolism disorders, hemoglobinopathies and abnormally increased stem cell dividing frequencies. In the first two cases hemoglobin concentration and mean corpuscular hemoglobin content (MCH) are decreased. Iron deficiency can result from impaired iron uptake or abnormal iron distribution, both resulting in changes of ferritin and transferrin values.

As demonstrated by these two examples, there are numerous connections between different parameters that have to be considered when blood values are interpreted. Therefore the systemic screening of a broad spectrum of different parameters provides a good basis to recover also small genotype effects and to identify possible reasons for the changes detected.

2.7 Immunological and Allergological Phenotyping

The pathophysiology of many diseases crucially involves cells and effector molecules belonging to the immune system. Therefore, phenotypical screening for immunodeficiencies within the German Mouse Clinic is central to identifying novel gene functions related to human diseases.

For primary screening, blood samples are analyzed in the Immunology unit at the GMC for the presence of leukocyte populations and immunoglobulin isotype classes. Together with the results from other screens this procedure should allow us to identify most mutant mouse lines with spontaneous immunodeficiencies. Additional protocols for systematic organ-specific analyses (flow cytometry of spleen, thymus and lymphnodes) have been established, which can be performed for secondary screening experiments. However, several immune functions will only be detectable upon stimulation. Therefore, we extended our analyses to *Listeria monocytogenes* infection.

2.7.1 Analysis of Flow Cytometric Phenotyping Data

The analysis of peripheral blood via flow cytometry is used as a tool to detect changes in the immune system. We have established a multi-parameter (12 parameters; 10 colors) flow cytometry-based protocol enabling us to identify all major leukocyte lineages with just two staining samples of peripheral blood. Moreover, we characterize T cell subsets regarding their expression of CD25, CD44, LY6C and CD62L. To determine the proportions of the main lineages in the peripheral

blood under high throughput conditions the implementation of an anti-CD45 antibody within a protocol that includes erythrocyte-lysis and live-dead discrimination with propidium iodide has been found advantageous.

In order to set the correct gates for positively stained cells, "fluorescence minus one" (FMO; Roederer, 2001) stainings are performed for each single marker within the antibody panel. The creation of an analysis template based on these FMOs allows a semiautomatic analysis.

The frequency of each subset is given as the proportion of the referring parent population. For main lineages, such as CD4+ and CD8+ T cells, B cells, NK cells, granulocytes and monocytes, the "all leukocyte gate" CD45+ is used as the parent gate. For T cell B cell subsets the CD4 and CD8 T and B cell clusters respectively are used as the parent gate.

Under baseline conditions the proportions of the main lineages, as well as T and B lymphocyte subsets, show relatively constant values within analyses of samples from the same line (Petkova et al., 2008, Chen and Harrison, 2002). FACS-analysis software like FlowJo® have already integrated statistical tests like Student's T and Wilcoxon Rank Tests for the comparison of each leukocyte subset from two different groups of samples. This software groups the samples based on their "keywords". We label each sample with keywords such as "mouse I.D. number", "sex" and "genotype" which are added to the layout template of the acquisition software (Diva®). In the interest of high throughput this is rapidly executed with the aid of a barcode scanner.

We have observed that differences between two groups of mice in the frequency of one leukocyte subset are linked with typical changes in the frequency of other subsets. Such correlations are found between the frequencies of CD4 and CD8 T cells (positively correlated), as well as between the frequencies of granulo-cytes and the frequencies of B or T cells (negatively correlated) and characterize also differences between different batches of wild type mice. Some interrelations occur with parent populations to subsequently gated subpopulations. For example, the frequency of T cells is most often positively correlated to the frequency of CD62L− expressing cells within the T cell cluster. At the level of T cell subsets we observe a strong interaction between CD4 and CD8 subsets. It therefore makes sense also to use methods of multivariate analysis for the comparison of different experimental groups of mice. The software "Spice" (Roederer, Nozzi & Nason, 2011) proved to be a useful tool for multivariate analysis especially for Boolean-gated subpopulations. We further implemented cluster analysis, arraying datasets of mice with similar patterns into clusters. This kind of analysis enabled us to find complex phenotypes in mutant mice that occurred with a low penetrance. In such cases, averaging the proportions of leukocyte subsets of a group of mice may not appropriately characterize their phenotype.

2.7.2 Immunoglobulins

Several human cancers and genetic diseases are associated with a change in the levels of immunoglobulins in the blood, and immunoglobulin deficiency syndromes

are among the most prevalent primary immunodeficiency diseases. Moreover, during the process of class-switch, somatic hypermutations occur which explains that genes related to recombination have been found to be correlated to defects in the immunoglobulin production.

In order to face the challenge of high throughput analyses of the levels of immunoglobulins in mouse blood plasma, we developed a Luminex bead-array for the measurement of the immunoglobulin isotypes IgM, IgA, IgG1, IgG2a and IgG2b, using the commercially available Bio-Plex® Bead coupling system (Biorad®) for the preparation of the beads. Our bead-array allows us the simultaneous quantification of the different immunoglobulin isotypes within a 1:1,000 diluted plasma sample at low costs with high accuracy.

Because of the extremely low plasma concentration of IgE under naïve conditions, this isotype cannot be detected within our multiplex test system. Therefore, we perform the classical sandwich ELISA to measure the IgE concentration in the 1:5 diluted mouse plasma samples. Levels of immunoglobulins in plasma have been shown to be linked to age. IgE concentrations are in addition linked to the sex, being higher in female mice (Alessandrini et al., 2001). Changes in levels of IgE can be associated to allergic diseases, parasitic infections and human syndromes such as Wiskott-Aldrich Syndrome (e.g., Kjellman, Johansson & Roth, 1976; Halonen et al., 1982; Villareal et al., 1999; Waldman et al., 1972).

2.8 Mouse Phenotyping by Steroid Quantification

Steroids control processes like differentiation and proliferation of cells and tissues. They further participate in regulation of inflammation (Hardy et al., 2008), apoptosis (Bansal, Houle & Melnykovych, 1991), bone remodeling (Jerome, 2004) or neuroregeneration (Chowen, Azcoitia, Cardona-Gomez & Garcia-Segura, 2000). Defects in steroid metabolism contribute as well to pathogenesis of complex diseases like diabetes, cancer, polycystic ovary syndrome, diseases of cartilage and bone, or neurological diseases (Mindnich & Adamski, 2007; Möller & Adamski, 2006; Tomlinson & Stewart, 2007). Altered relations of steroid concentrations are typical signs for steroid-related disorders. To routinely quantify steroids, we adopted ELISA (enzyme-linked immunoadsorbent assay) for measurements in rodents.

For screening of alterations in plasma steroid concentrations, we followed the procedure already described in (Gailus-Durner et al., 2009). Up to now, ELISA kits for dehydroepiandrosterone, testosterone, progesterone and estradiol (from Assay Designs and DRG Diagnostics) are customized for measurement of mouse samples. Further kits are available to determine estrone, androstenedione, aldosterone, dehydroepiandrosterone-sulfate, dihydrotestosterone, estriol, pregnenolone, and 17-hydroxyprogesterone on request. However, for these steroids the pre-ELISA-sample new preparation protocols are to be established to meet plasma demand and matrix effects.

Although steroid quantification is routinely performed in clinical chemistry for human samples, for mouse samples these determinations represent a major

challenge. Detection and quantification of steroids can be done by several technologies including RIA, GC/MS, LC/MS, and ELISA. RIA is a very sensitive method of quantification, but associated with radioactive exposure of people and environment. GC/MS is often applied for human samples. When applied to screening measurements of mouse plasma, the main limitations are due to minute sample volume and matrix effects (Prehn et al., 2007). The latest technology developments increase the potential of LC/MS-methods for steroid quantification (Haller, Prehn & Adamski, 2010). However, the technology is still cost-intensive and not standardized worldwide. At the moment, ELISA is our method of choice for steroid screening in mouse plasma, because of its standardization, reasonable costs and sound interpretation. Although the additional plasma extraction step is a drawback with respect to workload, this procedure results in samples with less matrix effects and enables an enrichment of steroids that are at the limit of detection in mouse plasma (e.g. estradiol).

When working with ELISA one should be aware that every single steroid needs its own measurement. This means, a larger amount of plasma is needed for multiple steroid testing/measurements. Furthermore, in some cases steroids are below the limit of detection. For example, estradiol cannot be quantified by ELISA in plasma of normal male mice. On the other hand, ELISA assays were successful in our steroid screen as the feminization of a transgenic mouse line was easily detected by reduced concentrations of testosterone in mutant male mice (manuscript in preparation).

2.9 Mouse Phenotyping by Targeted Metabolomics

Metabolomics is a very promising research field for phenotyping of biological samples with an unbiased approach of characterization. Especially, not manifested phenotypes (e.g., not visible in dysmorphology analyses) or subsidiary phenotypes (e.g., specific to one metabolic pathway but compensated by others) can be determined when many different parameters are correlated (Altmaier et al., 2009; Gieger et al., 2008; Illig et al., 2010; Wang-Sattler et al., 2008). Furthermore, very early metabolic responses to pharmacological treatment of mice can be (unequivocally) detected (Altmaier et al., 2008). Two major approaches are followed in the field of metabolomics: (1) targeted metabolomics, where chosen sets of metabolites are quantified and (2) non-targeted metabolomics aiming at metabolite identification and profiling.

Metabolomic phenotyping of mouse lines as offered by the German Mouse Clinic is done at the Genome Analysis Center of the Helmholtz Zentrum München by targeted metabolomics. Description of sample preparation and assay procedures used is available at http://www.helmholtz-muenchen.de/gac. Selected metabolites are quantified by two different kits, the Biocrates Absolute*IDQ*^{TM} p150 kit and the Chromsystems Newborn Screening kit, which together allow for the quantification of 163 and 42 endogenous metabolites in plasma and whole blood, respectively. These metabolites include amino acids, hexoses and lipids.

Both methods are established for high throughput analysis. Anyhow, to obtain comparable and reliable results it is very critical to strictly follow the Standard Operation Procedures (SOPs). As the sample amounts are not really limiting (20–30 μL of blood per single analysis are needed) these methods are also suitable for experiments in which a time course for a single mouse shall be followed.

The targeted metabolomics methods were already successful applied to the above mentioned (2.8) feminized transgenic mouse model. The metabolite pattern of feminized male mice was different to wild-type males but resembled that of female mice (manuscript in preparation). Another study shows that we can easily differentiate the metabolite patterns of mice of different sex or age (manuscript in preparation). Beside this, the methods are complementary to other "omics" methods like transcriptomics or genome wide association studies (Illig et al., 2010).

2.10 Cardiovascular Screening

Primary cardiovascular screening includes tail-cuff blood pressure analysis, a non-invasive measurement from the caudal artery of the tail in conscious mice, and examination of gross heart morphology and heart weight in relation to tibia length or body weight. Additional embedded secondary examinations can be performed if necessary including electrocardiography (ECG), echocardiography and determination of the atrial natriuretic peptide levels in plasma (as Nt-proANP) by immunoassay (Gailus-Durner et al., 2009).

Non-invasive blood pressure measurement serves as a potent tool for high throughput screening although the method includes some challenges. To accommodate the biological variability of blood pressure it is necessary to measure over several days. A mild stress by handling cannot effectively be overcome by training (Balcombe, Barnard & Sandusky, 2004) but has to be kept in mind as influencing factor. Nevertheless it is helpful to habituate the animals at least for one day prior to the measurement period to reduce the stress of unpredictability due to an unknown situation (Miczek, 1999). For a high throughput workflow we therefore decided to exclude the day of first exposure to the measurement and proceed with four continuous days of measurement. With this protocol we found alterations of blood pressure or pulse rate in 30% of mutant mouse lines. Data reveal a good robustness with reproducible differences in either the same line or lines with mutations in the same gene.

Despite the robustness of the values, in the 130 lines measured in our screen until now as well as within several studies of other investigators even pronounced differences are reflected by alterations around 30 mmHg (see e.g., Billet et al., 2007, Sugiyama, 1997). In our hands the only animals exceeding these ranges were strongly kidney-insufficient and dying within 3 month of age (unpublished data).

Another challenge within this measurement is that blood pressure and heart rate are integrated physiological phenomena that are influenced by many systems and processes and therefore affected in numerous disease syndromes. To perform the measurement within a systemic approach like the GMC is vital for a parameter as

blood pressure since it allows us the essential differential diagnosis on the basis of other screening results. Performance of our additional cardiac-specific paradigms enables us to recognize alterations of cardiac origin or, if a secondary etiology can be excluded, to identify models for primary hypertension that are extremely valuable for basic vascular research.

2.11 Systematic Mouse Phenotyping in Search of New Gene Candidates Playing a Role in Energy Homoeostasis

The regulation of body mass and endogenous energy reserves is an accurately tuned process based on a complex network of neuro-endocrine signaling cascades steering physiological pathways that are essential for vital metabolic functions whereas dysfunctional energy regulation is likely to result in serious metabolic disorders such as obesity and the metabolic syndrome.

In a vivid illustration, Hervey (1969, referring to an unpublished lecture by R. Passmore) pointed out that a seemingly insignificant excess consumption of 350 mg of food per day over a period of 40 years results in increased body mass by roughly 11 kg. Slightly re-phrasing this example while the total of all endogenous energy reserves of an adult human amounts to 700 MJ the daily energy flux under steady state conditions equals just 10 MJ turnover entering the organism via food (i.e. energy intake) or being allocated to the various components of the energy expenditure budget (i.e. activity, thermogenesis, tissue synthesis, reproduction, basal metabolism, diet induced thermogenesis) (Blouchard & Tremblay 1997). Rather small uncompensated irregularities in the ratio between caloric needs and the sustained excess intake of energy may result in serious obesity associated with deleterious health problems.

Morbid obesity and related disorders require medical treatment and due to its increasing prevalence all over the world extensive research efforts are being made to decipher gene functions related to metabolic disorders. A majority of this work has been conducted using suitable mouse models with the aim to monitor the functioning of energy regulation in genetically modified animals. Due to the allometric relationship of metabolic rate and body mass the comparison of humans and mice reveals that in mice the ratio between daily energy flux and total body energy reserves is considerably higher. As an example an average 12 weeks old mouse with a body mass of 22.3 g, fat content of 5.5 g and 13.3 g lean mass contains about 270 kJ body energy content. Daily energy expenditure amounts to about 39 kJ. In this model calculation energy turnover amounts to 1:7 of the body energy content which is much higher in mice compared with humans where this ratio is about 1:70. It can be concluded that gain or loss of body mass in mice requires larger changes in energy intake and/or energy expenditure which significantly facilitates the detection of disturbances in energy balance regulation

Systematic metabolic phenotyping of mouse models in search of new candidate genes involved in energy balance regulation usually comprises the measurement of a set of variables that are directly or indirectly related to energy flux. The rate of

energy turnover can be investigated both regarding energy intake (i.e. food consumption and processing) or energy expenditure (e.g. via oxygen consumption in an indirect calorimetry system). A sound diagnosis of irregularities between both sides of the energy balance equation, however, demands a sufficiently long period of time for the monitoring of a cohort of individual mice. If possible, energy intake should be determined in parallel with energy expenditure to assess imbalanced energy homoeostasis. Both rather static views on energy regulation described above do not take into account counter-regulatory energy dissipation mechanisms (Levine et al., 2005). Therefore, monitoring of both sides of the energy balance equation obtains more specific information on the metabolic phenotype.

Of course, this seems more feasible during the very dynamic phase of body mass changes e.g., immediately after inducing obesity by feeding a high caloric diet or in inducible knock out models. Clearly, as the capacity in a phenotyping facility designed for maximal throughput is limited, short-term measurements are more desirable. One must question whether small effects, i.e. increments of excess energy that result in a positive energy balance and obesity can be detected during short periods of experimentation.

And which metabolic traits are most informative in the search for new gene functions for energy balance regulation? First of all, differences in body mass and shifts in body composition provide important information on genotype effects on energy metabolism variables in mutant mouse lines. In a differential diagnosis including supporting results from clinical chemistry, dysmorphology, and behavioral screens a first-line indication for metabolic phenotypes may be evident. Based on our experience in mouse phenotyping this may not be sufficient to select the mutated gene as candidate gene for more detailed analysis of the mechanisms involved. Therefore, more specific tests are required to carve out the effects of the mutation on energy metabolism.

Indirect calorimetry is one way of collecting more specific data on energy homeostasis of mutant mice. However, as energy metabolism variables are related to body size and body mass disturbed energy flux itself must be evaluated taking into account body mass differences between mutant and wild-type mice. Simple mass-specific normalization of energy intake or energy expenditure will produce artifacts regarding hyper- or hypometabolism in mutant mice (Meyer, Klingenspor, Rozman & Heldmaier, 2004). Therefore, mass-specific energy turnover rate can be misleading when comparing genotypes and does not provide conclusive information on the ratio between energy intake and energy expenditure. How can we then extract meaningful information from short-term measurements of energy flux? We suggest that a linear regression model (LM) including body mass as covariate can be employed to specify gene functions affecting energy regulation (e.g., mean oxygen consumption). This approach was exemplified in *Umod*^{A227T} mice a mutation causing impaired kidney function but as a pleiotropic effect also resulting in reduced body mass. *Umod*^{A227T} mice were hypometabolic when metabolic rate was adjusted to body mass using a LM, and in addition showed reduced rectal body temperature thus confirming that the gene affected energy balance regulation, in addition to the well described kidney phenotype (e.g., Kemter et al., 2009). Using

this approach we have meanwhile identified several mutant lines with disturbances in energy balance. Apart from irregular locomotor activity or feeding patterns which are monitored in parallel with indirect calorimetry trials and can be used for phenotyping another diagnostic measure provided important information on energy metabolism. We found interesting deviations in the ratio between volumes of inhaled O_2 and exhaled CO_2, the respiratory exchange ratio (RER = VCO_2/VO_2) in some mutant lines indicating a shift in the substrates of metabolic fuel utilization. The underlying metabolic alterations causing these metabolic deviations require further investigation.

In conclusion, it is important to emphasize that the short-term measurement of energy flux in mouse mutants generates information that is very useful to identify metabolic phenotypes. Beyond the technical demanding set-up, data analysis issues require careful consideration to avoid misleading if not false diagnosis of genotype effects on energy homoeostasis.

2.12 Diabetes Mouse Phenotyping

Diabetes mellitus derives from Greek "to flow through like a siphon" and "honey" and entitles a chronic metabolic disorder of multiple etiology. In the first or second century A.D. the Greek physician Aretaeus "the Cappadocian" rendered the earliest clear accounts of diabetes for patients suffering from polyuria and excessive thirst and "siphoned or melted down flesh and limbs into the urine". Almost 2,000 years later diabetes has advanced to a global health issue and last year the International Diabetes Federation (IDF) predicted a rise to 285 million diabetics, equivalent to 6.4% of the world's population (IDF 2009). Due to the increasing need of therapeutic targets, to better understand diabetes and its complications, to identify new mechanisms, and to test novel treatment options the German Mouse Clinic (GMC) Diabetes Screen was established linking murine pathophysiology to human disease. The GMC Diabetes Screen provides an array of standardized procedures, scientific expertise, and state-of-the-art technologies for characterization of in vivo glucose homeostasis, insulin action, pancreatic β-cell function, organ pathology, and other physiological or anatomical alterations in mouse models.

2.12.1 Diabetes Pathophysiology and Gene-Environment Interactions

It is generally accepted that in most forms of diabetes multiple genetic and environmental components interact (Gerich, 1998; Reaven, 1995). Insulin is the major player and principal hormone regulating glucose uptake from blood into most cells (primarily muscle and fat cells) of the body. If in response to stimuli insulin is insufficiently released from pancreatic β-cells, if pancreatic β-cells are subject to destruction, or if cells become insulin resistant (displaying decreased sensitivity or responsiveness to insulin) blood glucose is inadequately utilized resulting in

persisting high blood glucose concentrations (hyperglycemia). Hyperglycemia is common to all forms of diabetes as well as derangements in lipid and protein homeostasis. Complex interactions of multiple environmental and genetic factors pose real challenges impeding the identification of exact molecular mechanisms implicated in diabetes pathophysiology and its treatment in human. Like the human, the mouse is a mammal with related physiology, anatomy, and developmental stages. Options such as targeted or non-targeted genetic manipulations, inbreeding, its small size, high reproductive rates (in many strains), and economic considerations advanced the mouse to a key model organism in biomedical research.

2.12.2 The German Mouse Clinic Diabetes Screen

Aims of the GMC Diabetes Screen are to (I) link murine pathophysiology to human disease, (II) better understand diabetes and its complications, (III) identify new mechanisms and therapeutic targets, and (IV) test novel treatment options. The number of engineered mouse mutants has steeply risen in recent years and simultaneously the demand for phenotyping mouse mutants with specialized protocols, assays, technologies, and exposed to environmental challenges. The GMC Diabetes Screen provides an array of standardized procedures, scientific expertise (Neschen, Morino, & Hammond, 2005; Jürgens, Neschen, & Ortmann, 2007; Neschen, Katterle, & Augustin, 2008, Morino, Neschen, & Bilz, 2008; Danial, Walensky, & Zhang, 2008), consultation in experimental setup and data interpretation to research users, and state-of-the-art technologies to characterize in vivo glucose homeostasis, insulin action, pancreatic β-cell function, organ pathology, and other physiologic or anatomic alterations in mice. Key to investigating a multifaceted disorder such as diabetes and its complications are close interactions between the Diabetes and the other 14 GMC disease screens (Gailus-Durner et al., 2005).

To evaluate the specific roles of gene products and their contributions to disease onset and development, potentially diabetes-prone mouse mutants are screened in the GMC Diabetes Screen. The glucose clamp technique – valuable in human research – is applied to conscious mice in a miniaturized format and employed for quantification of systemic and localizing organ-specific alterations in insulin action (euglycemic-hyperinsulinemic clamps) or pancreatic β-cell function (hyperglycemic clamps; DeFronzo, Tobin & Andres, 1979). The following examples from mouse mutants exerting an initially unexpected diabetes phenotype outline that diabetes screening enables novel insights into gene regulation in diabetes, diabetes development, and its pathogenesis.

Associations between chronic low-grade inflammation, obesity, and insulin resistance are recognized for several years (Zeyda & Stulnig, 2009) but the specific underlying mechanisms remain unclear. Experiments carried out by collaboration partners indicated that mice homozygous for a targeted mutation in a gene encoding a glycoprotein affecting inflammation, cell attachment, and tissue remodeling were protected from deteriorating insulin tolerance when fed a high-fat diet. Based

on these initial findings high-fat diet-induced obese, age-, and weight-matched mutant and wild-type mice were subjected to euglycemic-hyperinsulinemic clamps combined with a radioisotope approach (to estimate glucose fluxes) in the GMC Diabetes Screen with the aim to localize so far unknown organ-specific alterations in insulin action. Data confirmed increased whole-body insulin sensitivity in mutant mice suggesting improved hepatic (but neither skeletal muscle nor adipose tissue) insulin sensitivity did account for this effect (unpublished data).

Glucose-stimulated insulin secretion (GSIS) from pancreatic β-cells is the pivotal homeostatic process controlling blood glucose levels (Ashcroft & Rorsman, 2004; Cerasi, 1975). Understanding the underlying biochemical mechanisms would be highly desirable for the development of diabetes therapies. A novel putative gene target has been recently identified in an iron storage disease mouse model. Mutant mice tested by the GMC Clinical Chemistry and Metabolism Screens exhibited a marked reduction in glucose tolerance compared to wild-type controls. When subjected to hyperglycemic clamps in the GMC Diabetes Screen mutant males displayed markedly lower plasma insulin concentrations both, under fasting conditions and in response to persisting physiological hyperglycemia compared to wild-type (data unpublished). To whether the hypothesis, that the particular gene product alters mitochondrial respiration and tricarboxylic acid cycle activity thereby affecting insulin secretion from pancreatic β-cells is accepted, is under investigation.

2.12.3 Environmental Challenges in the Diabetes Screen

A substantial proportion of type 2 diabetes, constituting the vast majority among diabetic subjects, can be prevented or its onset delayed (IDF 2009). Effective therapeutic measures are, e.g., overweight reduction, increase of physical activity, and the use of certain glucose-lowering drugs.

Design and application of customized environmental challenges that take the type of genetic modification and its putative consequences in disease into account, is an important facet of the GMC Diabetes Screen. The spectrum ranges from experimental diets high in selected fatty acids capable of inducing fatty liver (and fat-induced hepatic insulin resistance) to such composed to induce/accelerate/prevent obesity or pancreatic β-cell atrophy. A major strength of the challenge platform at the GMC is the option to design allotropic and complex environmental challenges (e.g., chronic treadmill exercise combined with cafeteria diet regimen). Furthermore “tailor-made” challenge experiments in mutant mouse lines with anti-diabetic agents to better understand compound action and disease mechanisms that are part of the GMC Diabetes Screen program. The potential role of pharmacologic agents in treating particular diabetes pathologies and its complications will soon represent a further core task. Worth noting is the fact that no mouse model will ever exactly match human diabetes characteristics. But certain mouse models are better suited for analyzing particular effects than others. Key to designing a study is keeping in mind a mouse models ascribed genetic, endocrine, metabolic, and pathologic (hyperglycemia, insulin resistance, hyperinsulinemia,

β-cell destruction, obesity, etc.) condition and to account for that when designing experiments. The "best choice" mouse model will then serve as an essential tool for identifying new targets in the treatment of certain aspects of human diabetes and its complications.

2.13 The Introduction of the Nuclear Magnetic Resonance Technology at the German Mouse Clinic

The German Mouse Clinic has recently acquired a Nuclear Magnetic Resonance system, equipped with a 9.7 T magnet, several radiofrequency coils for specific applications and optimized gradient coils for great efficiency of the scanning processes. Besides, it is comprised of auxiliary software and particular accessories to facilitate investigations in mice. The imaging itself delivers excellent resolution and soft tissue differentiation. It can be performed multiple times at the same animal to gain knowledge about the temporal changes of the anatomy in its development.

The NMR spectroscopy is a powerful tool and the system can be used by various screens of the German Mouse Clinic because it enables the anatomic scanning of several organs like brain, cartilage, live and heart as well as the visualization of physiological processes by means of ^{1}H spectroscopy and chemical shift imaging. This allows monitoring of dynamic events such as the lipid metabolism. These methods will help to understand mechanisms of various diseases like degenerative changes of the nervous system, cardiovascular diseases, and diabetes. The NMR analysis of mouse models will help to analyze how close the model is to the human counterpart.

One of the initial aims for the use of the Nuclear Magnetic Resonance in the German Mouse Clinic is to analyze dysfunction in pancreatic cells caused by diabetes mellitus (Aigner et al., 2008). Biochemical changes in these tissues can be investigated by chemical shift imaging, which allows the study of various types of biochemicals common in the body (such as water, fat, choline, n-acetyl aspartate, lactate, etc.). Another aim is to examine heart malformations and dysfunctions caused as a secondary effect of the Osteogenesis imperfecta disease (Lisse et al., 2008). By cardiac scanning synchronized with the electrocardiograph and respiratory cycles, it is possible to determine morphology and dynamics of three-dimensional heart structures.

For these and all other applications of phenotypic characterization by NMR the system uniquely allows the German Mouse Clinic to perform highest quality high throughput screening of mice.

2.14 Molecular Phenotyping

The molecular phenotyping screen focuses on the analysis of gene regulation in mouse models for human inherited diseases. Such systematic analysis of mRNA expression levels of mouse mutants supports the understanding of the underlying

molecular biology in these mutant mouse models and provides new insight into mammalian gene function (Nadeau et al., 2001; Beckers & Hrabě de Angelis, 2002). In addition, these analyses reveal target genes directly or indirectly regulated by a genetic modification, affected molecular pathways, groups of co-expressed genes, and potential targets for therapy.

In the primary screen a set of organs is systematically collected for each mutant mouse line submitted to the GMC of age-match male mice (Horsch et al., 2008). Organs for genes expression profiling are selected based on conspicuous phenotypes in other GMC screens. Alternatively, the selection is based on previous knowledge about mutant phenotypes or based on known or predicted gene functions. Differential gene expression is identified in 53% of the analyzed organs. According to the current numbers, gene regulation was frequently detected in brain, spleen, thymus, liver and muscle. In contrast, identification of gene regulation was rather rare in heart, kidney or testis. In 60% of the organs selected due to previous knowledge of gene function significant gene regulation was detected. 51% of those organs chosen based on conspicuous phenotypes in at least one other GMC screen showed changes in their gene expression levels. To conclude, both strategies of organ selection are highly efficient for the identification of gene regulation (Horsch et al., 2008). In a secondary screen organs are analyzed that are not included in the routine set of the primary screen or that are collected from animals after environmental challenges (e.g., stress, nutrition [Bender et al., 2007], infection, or air pollution [Maier et al., 2008a]).

A reference database of expression profiles was constructed by the import of all microarray experiments in the GMC database "MausDB" (Maier et al., 2008b). By pattern matching we can use the database for classification of the analyzed mutant mouse lines and the characterization of novel mutants by the identification of global profile similarities.

2.15 The Histopathological Phenotyping of Mouse Models of Human Diseases in the German Mouse Clinic

The Pathology Screen in the German Mouse Clinic performs a detailed morphology-based analysis of mutant mouse models to examine genotype-induced alterations bearing in mind mouse background strain, health and environmental influences. This evaluation plays a vital role in a multidisciplinary effort to characterize mutant mouse models and provide insights into the understanding how genetic alterations influence the development of human diseases that the mutant mice are intend to model. Here we present an overview of (1) the elaborated work carried out in our screen, based on the knowledge of mouse histology among different mouse strains and of health status influences, (2) the importance in correlating morphological tissue alterations with the findings derived from other screens. The parallel that can be established between mouse and human diseases is exemplarily shown.

The development of the genetically engineered mice has provided a considerable variety of mice models of human diseases, which facilitate the examination of

normal and altered regulation of gene expression and cellular processes. The identification of morphological abnormalities on the tissue level and its correlation with other screens (e.g. clinical chemistry, immunology, allergy and neurology) translate into a collective effort to perform a complete phenotypic evaluation of mouse models pointing to which organ system(s) is being primarily affected by the mutation of interest.

2.15.1 Comprehensive Pathological Assessment

The primary screen of the pathology screen elaborates for each mouse line a standard and comprehensive protocol, which may include additional evaluations specific to the mutation of interest or requested by the mouse provider. The number of mice provided for evaluation comprehends generally 10 mutant mice of both sexes (17–20 weeks of age) and the same number of control littermates facilitating the genotype-derived characterization.

The standard procedures from blood removal, dissection of 30 tissue samples (http://eulep.pdn.cam.ac.uk/Necropsy_of_the_Mouse/index.php) to data recording are reproducibly performed as described in Moßbrugger, Hölzlwimmer, Calzada-Wack, and Quintanilla-Martinez (2007), thus ensuring a uniformed evaluation minimizing the intra-technician variability. These time-consuming procedures can be supplemented with radiography and photography when appropriate. Specific targeted evaluation can additionally be performed in a secondary screen such as quantitative immunohistochemistry and transmission electron microscopy, which provide further details into the histological findings.

Our screen generates enormous quantities of data for each mutant mouse that range from body and organ weights, photos from gross morphology (lesions, tumors) and from the histological diagnosis itself. The data is placed in a data bank with the aim to be freely accessed by the scientific community promoting therefore on line discussions and conferences worldwide.

2.15.2 Background Strain Influences

In recent years the pathology screen in our laboratory has gained a vast experience in strain-related histological alterations. The most used background stains in our screen are C57BL/6, C3H, BALB/c and 129/Sv. These strains present variations in organs such as adrenal glands, intestine, liver and have been well characterized by our group (Moßbrugger et al., 2007). For instance, microgranulomas are often found in the liver of C57BL/6 mice, the corpus callosum is missing in almost 50% of BALB/c mice and > 50% of 129/Sv mice, the bronchial associated lymphoid tissue (BALT) is very prominent in C3H mice compared with other strains. The knowledge of such strain-related alterations minimizes therefore false diagnosis and overall improves the quality and reliability histopathological evaluation.

2.15.3 Relation of the Pathology with Other Screens

The German Mouse Clinic was conceived to provide the scientific community with a complete, systematic characterization of mouse models in which 14 distinct screens are performed. The pathology screen is the last one to be performed in the workflow but may be the one to corroborate or even clarify the functional changes observed in other screens. We recently worked with a mouse model that exhibited phenotypes for clinical chemistry, behavior, steroidogenesis and energy metabolism and we were able to diagnose a 100% penetrance of an adrenal gland phenotype. In this case, we observed an atrophic adrenal cortex and an extensive vacuolization of the adrenal medulla. Another example of the interdisciplinary work performed in the mouse clinic concerns a mutant mouse line presenting hydronephrosis and increased plasma urea concentrations.

2.15.4 Mouse Histopathology: Mirroring Human Conditions

From the mutant lines recently analyzed in the pathology screen, 42% showed a pathological phenotype, 31% being newly identified. Twenty one percent of the lines with a pathological phenotype had pathological alterations of the skeletal system. A neurological and lymphatic system phenotype was seen in 19 and 14% of these mouse lines, respectively. Nine percent of the lines exhibiting tumors, while the remaining lines presented alterations in vital organs such as heart, liver, lung, kidney, pancreas and adrenal glands. It is interesting to note that many of these models mimic the human disease and provide direct comparisons with human histopathology. On the other hand, it is important to bear in mind the differences. Transgenic models of neurodegenerative diseases, such as Parkinson's or Alzheimer's disease display degenerative changes of neurons and a surrounding inflammatory reaction of the glial cells that mirror the human disease, but the morphology and composition of the characteristic inclusions and deposits vary widely, according to the underlying genetic mutation (Masliah et al., 2000, Roskam, Neff, Schwarting, Bacher, & Dodel, 2010, Rosemann et al., 2010). Additionally, certain diagnoses and terminology used in humans not always apply to mutant mice. In hematopoietic histological characterizations, a direct parallel cannot be drawn due to the fact that in adult mice the spleen is the primary hematopoietic organ (equivalent in many ways to the human bone marrow). In order to overcome such limitations, the Bethesda proposals for classification of nonlymphoid hematopoietic neoplasm (Kogan et al., 2002) are applied. In addition, the use of immunohistochemistry markers with standardized protocols established for the murine tissues (Kunder et al., 2007) facilitates the histological diagnosis and assists in this comparison. Several other classification schemes in organs such as mammary gland (Cardiff et al., 2000), nervous system (Weiss et al., 2002), intestinal carcinoma (Boivin et al., 2003), pulmonary (Nikitin et al., 2004), prostatic (Shappell et al., 2004) and pancreatic lesions (Hruban et al., 2006, Fig. 2.2) are also valuable guidelines for pathologists in the interpretation of neoplastic lesions in mouse models.

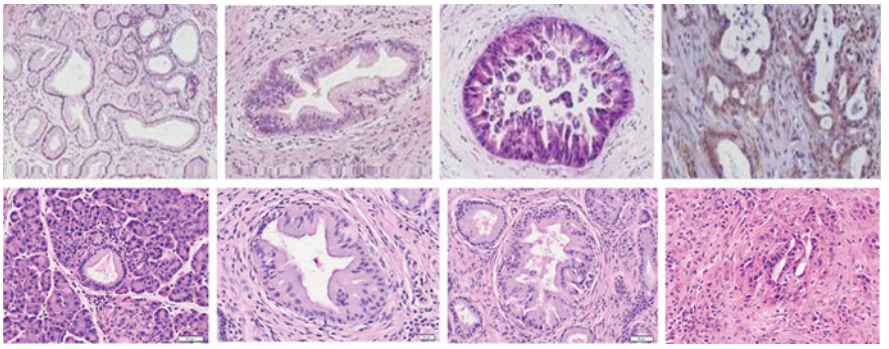

Fig. 2.2 Photomicrographs of histological pancreatic cancer precursor lesions designated as PanINs. *Panel above*: human lesions grouped in three histological stages based on increasing degrees of architectural and nuclear atypia, from left to right PanIN1, 2, 3 and pancreatic ductal adenocarcinoma; *Panel below*: comparable lesions observed in a mutant mouse model (*LSL-KRAS*$^{G12D/+}$; *Ptf1a-Cre*$^{ex1/+}$). The similar nomenclature of the lesions facilitates the comparison between mouse models and human pancreatic disease

2.16 Outlook

Within the GMC we have developed specific standardized challenge platforms to explore the complex relationship between environmental changes and genetic factors and to describe and define envirotypes (Fuchs et al., 2009; Beckers, Wurst & Hrabě de Angelis, 2009). By mimicking specific live styles or exposures we want to analyze physiological and molecular mechanisms of genome-environment interactions. Main focus is put on dietary and metabolic challenges (Bender et al., 2008; Rothwell & Stock, 1981; Haemmerle et al., 2006; Somel et al., 2008), physical activity (Hoshi, Watanabe, Chiba & Inaba, 1998, Lerman et al., 2002), different stress conditions (Lehtinen & Bonni, 2006; Uttara, Singh, Zamboni & Mahajan, 2009, Joo et al., 2009), inhalation of nanoparticles (Ganguly et al., 2009), host-pathogen interactions (Busch, Kerksiek & Pamer, 1999) and allergical challenges (Alessandrini et al., 2006). In the present systemic primary phenotyping screen the analysis is based on studying unchallenged mice in a protected, pathogen-free environment. To yield even more comprehensive information from the primary phenotyping analysis it will be necessary to implement challenge protocols. Only challenge tests that are easy to control and to perform can be incorporated in a systemic screen like specific dietary or fasting conditions. More time intensive challenge conditions might require the design of various systemic pipelines with specific disease focuses. Especially for an open-access platform like the GMC these developments mean to further customize the systemic primary analysis. In the future, new developments in the GMC will also involve the extension of systemic primary phenotyping by monitoring the influence of new compounds and drugs on mouse physiology.

To provide capacities for systemic phenotyping, archiving and distribution of mouse models for the biomedical research community in the future the

pan-European initiative Infrafrontier has built a network of phenotyping centers and archiving institutions. New mouse clinics will be established and new archiving nodes of EMMA (European Mouse Mutant Archive) will provide the capacities required by the scientific community for high-throughput phenotyping.

Acknowledgments We would like to thank Reinhard Seeliger, Miriam Backs, Ingrid Bayer, Nicole Boche, Sabrina Bothur, Sandra Geissler, Michaela Grandl, Tamara Halex, Elfi Holupirek, Sabine Holthaus, Constanze König, Maria Kugler, Albert Langer, Katrin Laube, Astrid Markert, Jacqueline Müller, Elenore Samson, Florian Schleicher, Daniela Schmidt, Waldemar Schneider, Ann-Elisabeth Schwarz, Bettina Sperling, Waldtraud Stettinger, Lucie Thurmann, Susanne Wittich, and Claudia Zeller as well as the GMC animal caretaker team for expert technical help. This work has been funded by the BMBF (DZD e.V. 01GI0923 and NGFNplus grant, 01GS08156, 01GS0850, 01GS0851, 01GS0852, 01GS0868, 01GS0869, 01GS0853, and 01GS0854) and by an EU grant (EUMODIC, LSHG-2006-037188, German Mouse Clinic).

References

Abe, K., Fuchs, H., Lisse, T., Hans, W., & Hrabě de Angelis, M. (2006). New ENU induced semidominant mutation, Ali18, causes inflammatory arthritis, dermatitis, and osteoporosis in the mouse. *Mammalian Genome*, *17*(9), 915–926.

Abe, K., Wechs, S., Kalaydjiev, S., Franz, T. J., Busch, D. H., Fuchs, H., et al. (2008). Novel lymphocyte-independent mechanisms to initiate inflammatory arthritis via bone marrow-derived cells of Ali18 mutant mice. *Rheumatology*, *47*(3), 292–300.

Aguilar-Pimentel, J. A., Alessandrini, F., Huster, K. M., Jakob, T., Schulz, H., Behrendt, H., et al. (2010). Specific CD8 T cells in IgE-mediated allergy correlate with allergen dose and allergic phenotype. *American Journal of Respiratory and Critical Care Medicine*, *181*(1), 7–16.

Aigner, B., Rathkolb, B., Herbach, N., Hrabě de Angelis, M., Wanke, R., & Wolf, E. (2008). Diabetes models by screen for hyperglycemia in phenotype-driven ENU mouse mutagenesis projects. *American Journal of Physiology – Endocrinology and Metabolism*, *294*(2), E232–40.

Aigner, B., Rathkolb, B., Herbach, N., Kemter, E., Schessl, C., Klaften, M., et al. (2007). Screening for increased plasma urea levels in a large-scale ENU mouse mutagenesis project reveals kidney disease models. *American Journal of Physiology – Renal Physiology*, *292*(5), F1560–1567.

Alessandrini, F., Jakob, T., Wolf, A., Wolf, E., Balling, R., Hrabě de Angelis, M., et al. (2001). ENU mouse mutagenesis: Generation of mouse mutants with aberrant plasma IgE levels. *International Archives of Allergy and Applied Immunology*, *124*, 25–28.

Alessandrini, F., Schulz, H., Takenaka, S., Lentner, B., Karg, E., Behrendt, H., et al. (2006). Effects of ultrafine carbon particle inhalation on allergic inflammation of the lung. *Journal of Allergy and Clinical Immunology*, *117*(4), 824–830.

Altmaier, E., Kastenmuller, G., Romisch-Margl, W., Thorand, B., Weinberger, K. M., Adamski, J., et al. (2009). Variation in the human lipidome associated with coffee consumption as revealed by quantitative targeted metabolomics. *Molecular Nutrition & Food Research*, *53*(11), 1357–1365.

Altmaier, E., Ramsay, S. L., Graber, A., Mewes, H. W., Weinberger, K. M., & Suhre, K. (2008). Bioinformatics analysis of targeted metabolomics–uncovering old and new tales of diabetic mice under medication. *Endocrinology*, *149*(7), 3478–3489.

Ashcroft, F., & Rorsman, P. (2004). Type 2 diabetes mellitus: Not quite exciting enough? *Human Molecular Genetics*, *13*(Spec No, 1), 1221–1231.

Austin, C. P., Battey, J. F., Bradley, A., Bucan, M., Capecchi, M., Collins, F. S., et al. (2004). The knockout mouse project. *Nature Genetics*, *36*, 921–924.

Balcombe, J. P., Barnard, N. D., & Sandusky, C. (2004). Laboratory routines cause animal stress. *Contemporary Topics in Laboratory Animal Science*, *43*, 42–51.

Bansal, N., Houle, A., & Melnykovych, G. (1991). Apoptosis: Mode of cell death induced in T cell leukemia lines by dexamethasone and other agents. *FASEB Journal*, *5*(2), 211–216.

Baroncelli, G. I., Bertelloni, S., Ceccarelli, C., & Saggese, G. (1998). Measurement of volumetric bone mineral density accurately determines degree of lumbar undermineralization in children with growth hormone deficiency. *Journal of Clinical Endocrinology and Metabolism*, *83*(9), 3150–3154.

Beckers, J., & Hrabě de Angelis, M. (2002). Large-scale mutational analysis for the annotation of the mouse genome. *Current Opinion in Chemical Biology*, *6*(1), 17–23.

Beckers, J., Wurst, W., & Hrabé de Angelis, M. (2009). Towards better mouse models: Enhanced genotypes, systemic phenotyping and envirotype modelling. *Nature Reviews Genetics*, *10*(6), 371–380.

Bender, A., Beckers, J., Schneider, I., Hölter, S. M., Haack, T., Ruthsatz, T., et al. (2008). Creatine improves health and survival of mice. *Neurobiology of Aging*, *29*(9), 1404–1411.

Billet, S., Bardin, S., Verp, S., Baudrie, V., Michaud, A., Conchon, S., et al. (2007). Gain-of-function mutant of angiotensin II receptor, type 1A, causes hypertension and cardiovascular fibrosis in mice. *The Journal of Clinical Investigation*, *117*, 1914–1925.

Blouchard, C., & Tremblay, A. (1997). Genetic influence on the resonde of body fat and fat distribution to positive and negative energy balances in human identical twins. *Journal of Nutrition*, *127*, 943S–947S.

Boivin, G. P., Washington, K., Yang, K., Ward, J. M., Pretlow, T. P., Russell, R., et al. (2003). Pathology of mouse models of intestinal cancer: Consensus report and recommendations. *Gastroenterology*, *124*, 762–777.

Brooks, S. P., & Dunnett, S. B. (2009). Tests to assess motor phenotype in mice: A user's guide. *Nature Reviews Neuroscience*, *10*(7), 519–529.

Brown, S. D., Chambon, P., Hrabě de Angelis, M., & Eumorphia Consortium (2005). EMPReSS: Standardized phenotype screens for functional annotation of the mouse genome. *Nature Genetics*, *37*(11), 1155.

Busch, D. H., Kerksiek, K., & Pamer, E. G. (1999). Processing of Listeria monocytogenes antigens and the in vivo T-cell response to bacterial infection. *Immunology Review*, *172*, 163–169.

Cardiff, R. D., Anver, M. R., Gusterson, B. A., Hennighausen, L., Jensen, R. A., Merino, M. J., et al. (2000). The mammary pathology of genetically engineered mice: The consensus report and recommendations from the Annapolis meeting. *Oncogene*, *19*, 968–988.

Carlson, C. M., Dupuy, A. J., Fritz, S., Roberg-Perez, K. J., Fletcher, C. F., & Largaespada, D. A. (2003). Transposon mutagenesis of the mouse germline. *Genetics*, *165*(1), 243–256.

Cerasi, E. (1975). Mechanisms of glucose stimulated insulin secretion in health and in diabetes: Some re-evaluations and proposals. *Diabetologia*, *11*, 1–13.

Chen, J., & Harrison, D. E. (2002). Quantitative trait loci regulating relative lym-phocyte proportions in mouse peripheral blood. *Blood*, *99*(2), 561–566.

Chipman, S. D., Sweet, H. O., McBride, D. J., Jr, Davisson, M. T., Marks, S. C., Jr, Shuldiner, A. R., et al. (1993). Defective pro alpha 2(I) collagen synthesis in a recessive mutation in mice: A model of human osteogenesis imperfecta. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(5), 1701–1705.

Chowen, J. A., Azcoitia, I., Cardona-Gomez, G. P., & Garcia-Segura, L. M. (2000). Sex steroids and the brain: Lessons from animal studies. *Journal of Pediatric Endocrinology & Metabolism*, *13*(8), 1045–1066.

Collins, F. S., Finnell, R. H., Rossant, J., & Wurst, W. (2007a). A new partner for the international knockout mouse consortium. *Cell*, *129*(2), 235.

Collins, F. S., Rossant, J., Wurst, W., & International Mouse Knockout Consortium (2007b). A mouse for all reasons. *Cell*, *128*(1), 9–13.

Covelli V – Guide to the Necropsy of the Mouse. http://eulep.pdn.cam.ac.uk/Necropsy_of_the_Mouse/index.php

Crawley, J. N. (2008). Behavioral phenotyping strategies for mutant mice. *Neuron*, *57*(6), 809–818.

Danial, N. N., Walensky, L. D., Zhang, C. Y., Choi, C. S., Fisher, J. K., Molina, A. J. A., et al. (2008). Dual role of proapoptotic BAD in insulin secretion and beta cell survival. *Nature Medicine*, *14*, 144–153.

DeFronzo, R. A., Tobin, J. D., & Andres, R. (1979). Glucose clamp technique: A method for quantifying insulin secretion and resistance. *American Journal of Physiology*, *237*, G214–G223.

Enard, W., Gehre, S., Hammerschmidt, K., Hölter, S. M., Blass, T., Somel, M., et al. (2009). A humanized version of Foxp2 affects cortico-basal ganglia circuits in mice. *Cell*, *137*(5), 961–971.

Fuchs, H., Gailus-Durner, V., Adler, T., Pimentel, J. A., Becker, L., Bolle, I., et al. (2009). The German Mouse Clinic: A platform for systemic phenotype analysis of mouse models. *Current Pharmaceutical Biotechnology*, *10*(2), 236–243.

Fuchs, H., Schughart, K., Wolf, E., Balling, R., & Hrabé de Angelis, M. (2000). Screening for dysmorphological abnormalities – a powerful tool to isolate new mouse mutants. *Mammalian Genome*, *11*(7), 528–530.

Gailus-Durner, V., Fuchs, H., Adler, T., Aguilar Pimentel, A., Becker, L., Bolle, I., et al. (2009). Systemic first-line phenotyping. *Methods in Molecular Biology*, *530*, 463–509.

Gailus-Durner, V., Fuchs, H., Becker, L., Bolle, I., Brielmeier, M., Calzada-Wack, J., et al. (2005). Introducing the German Mouse Clinic: Open access platform for standardized phenotyping. *Nature Methods*, *2*, 403–404.

Ganguly, K., Upadhyay, S., Irmler, M., Takenaka, S., Pukelsheim, K., Beckers, J., et al. (2009). Pathway focused protein profiling indicates differential function for IL-1B, -18 and VEGF during initiation and resolution of lung inflammation evoked by carbon nanoparticle exposure in mice. *Particle and Fibre Toxicology*, *6*, 1725.

Gasser, J. A. (2003) Bone measurements by peripheral quantitative computed tomography in rodents. *Methods Molecular Medicine*, *80*, 323–341.

Gerich, J. E. (1998). The genetic basis of type 2 diabetes mellitus: impaired insulin secretion versus impaired insulin sensitivity. *Endocrine Reviews*, *19*(4), 491–503.

Giampietro, P. F., Blank, R. D., Raggio, C. L., Merchant, S., Jacobsen, F. S., Faciszewski, T., et al. (2003). Congenital and idiopathic scoliosis: Clinical and genetic aspects. *Clinical Medicine & Research*, *1*(2), 125–136.

Gieger, C., Geistlinger, L., Altmaier, E., Hrabé de Angelis, M., Kronenberg, F., Meitinger, T., et al. (2008). Genetics meets metabolomics: A genome-wide association study of metabolite profiles in human serum. *PLoS Genetics*, *4*(11), e1000282.

Green, E. C., Gkoutos, G. V., Lad, H. V., Blake, A., Weekes, J., & Hancock, J. M. (2005). EMPReSS: European mouse phenotyping resource for standardized screens. *Bioinformatics*, *21*(12), 2930–2931.

Guan, C., Ye, C., Yang, X., & Gao, J. (2010). A review of current large-scale mouse knockout efforts. *Genesis*, *48*(2), 73–85.

Haemmerle, G., Lass, A., Zimmermann, R., Gorkiewicz, G., Meyer, C., Rozman, J., et al. (2006). Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. *Science*, *312*, 734–737.

Hafezparast, M., Ahmad-Annuar, A., Wood, N. W., Tabrizi, S. J., & Fisher, E. M. (2002). Mouse models for neurological disease. *The Lancet Neurology*, *1*(4), 215–224.

Haller, F., Prehn, C., & Adamski, J. (2010). Quantification of steroids in human and mouse plasma using online solid phase extraction coupled to liquid chromatography tandem mass spectrometry Nature Protocols 10.1038/nprot.2010.22

Halonen, M., Barbee, R. A., Kholoitz, M. A., & Burrows, B. (1982). An epidemiological study of the interrelationships of total serum Immunoglobulin E, allergy skin-test reactivity and eosinophilia, J. *Journal of Allergy and Clinical Immunology*, *69*, 221.

Hansen, J., Floss, T., Van Sloun, P., Füchtbauer, E. M., Vauti, F., Arnold, H. H., et al. (2003). A large-scale, gene-driven mutagenesis approach for the functional analysis of the mouse genome. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(17), 9918–9922.

Hansen, G. M., Markesich, D. C., Burnett, M. B., Zhu, Q., Dionne, K. M., Richter, L. J., et al. (2008). Large-scale gene trapping in C57BL/6 N mouse embryonic stem cells. *Genome Research*, *18*(10), 1670–1679.

Hardy, R. S., Rabbitt, E. H., Filer, A., Emery, P., Hewison, M., Stewart, P. M., et al. (2008). Local and systemic glucocorticoid metabolism in inflammatory arthritis. *Annals of the Rheumatic Diseases*, *67*(9), 1204–1210.

Hervey, G. R. (1969). Regulation of energy balance. *Nature*, *222*, 629–631.

Horsch, M., Schädler, S., Gailus-Durner, V., Fuchs, H., Meyer, H., Hrabě de Angelis, M., et al. (2008). Systematic gene expression profiling of mouse model series reveals coexpressed genes. *Proteomics*, *8*(6), 1248–1256.

Hoshi, A., Watanabe, H., Chiba, M., & Inaba, Y. (1998). Effects of exercise at different ages on bone density and mechanical properties of femoral bone of aged mice. *The Tohoku Journal of Experimental Medicine*, *185*(1), 15–24.

Hough, T. A., Nolan, P. M., Tsipouri, V., Toye, A. A., Gray, I. C., Goldsworthy, M., et al. (2002). Novel phenotypes identified by plasma biochemical screening in the mouse. *Mammalian Genome*, *13*(10), 595–602.

Hrabě de Angelis, M., Flaswinkel, H., Fuchs, H., Rathkolb, B., Soewarto, D., Marschall, S., et al. (2000). Genome-wide, large-scale production of mutant mice by ENU mutagenesis. *Nature Genetics*, *25*(4), 444–447.

Hruban, R. H., Adsay, N. V., Albores-Saavedra, J., Anver, M. R., Biankin, A. V., Boivin, G. P., et al. (2006). Pathology of genetically engineered mouse models of pancreatic exocrine cancer: Consensus report and recommendations. *Cancer Research*, *66*, 95–106.

IDF (2009, October) Diabetes Atlas 4th edition, 20th World Diabetes Congress, Montreal, http://www.diabetesatlas.org/

Illig, T., Gieger, C., Zhai, G., Romisch-Margl, W., Wang-Sattler, R., Prehn, C., et al. (2010). A genome-wide perspective of genetic variation in human metabolism. *Nature Genetics*, *42*(2), 137–141.

Jerome, C. P. (2004). Hormonal therapies and osteoporosis. *Ilar Journal*, *45*(2), 170–178.

Joo, Y., Choi, K. M., Lee, Y. H., Kim, G., Lee, D. H., Roh, G. S., et al. (2009). Chronic immobilization stress induces anxiety- and depression-like behaviors and decreases transthyretin in the mouse cortex. *Neuroscience Letters*, *461*(2), 121–125.

Jürgens, H. S., Neschen, S., Ortmann, S., Schmolz, K., et al. (2007). Development of diabetes in obese, insulin-resistant mice: Essential role of dietary carbohydrate in beta cell destruction. *Diabetologia*, *50*(7), 1481–1489.

Karsenty, G., & Wagner, E. F. (2002). Reaching a genetic and molecular understanding of skeletal development. *Developmental Cell*, *2*(4), 389–406.

Kemter, E., Rathkolb, B., Rozman, J., Hans, W., Schrewe, A., Landbrecht, C., et al. (2009). Novel missense mutation of uromodulin in mice causes renal dysfunction with alterations in urea handling, energy, and bone metabolism. *American Journal of Physiology – Renal Physiology*, *297*(5), F1391–F1398.

Kjellman, N., Johansson, S. D. O., & Roth, A. (1976). Serum IgE levels in healthy children quantified by a sandwich technique (PRIST). *Clinical Allergy*, *6*, 51–59.

Kogan, S. C., Ward, J. M., Anver, M. R., Berman, J. J., Brayton, C., Cardiff, R. D., et al. (2002). Bethesda proposals for classification of nonlymphoid hematopoietic neoplasms in mice. *Blood*, *100*, 238–245.

Kunder, S., Calzada-Wack, J., Hölzlwimmer, G., Müller, J., Kloss, C., Howat, W., et al. (2007). A comprehensive antibody panel for immunohistochemical analysis of formalin-fixed, paraffin-embedded hematopoietic neoplasms of mice: Analysis of mouse specific and human antibodies cross-reactive with murine tissue. *Toxicologic Pathology*, *35*, 366–375.

Lehtinen, M. K., & Bonni, A. (2006). Modeling oxidative stress in the central nervous system. *Current Molecular Medicine*, *6*(8), 871–881.

Lerman, I., Harrison, B. C., Freeman, K., Hewett, T. E., Allen, D. L., Robbins, J., et al. (2002). Genetic variability in forced and voluntary endurance exercise performance in seven inbred mouse strains. *Journal of Applied Physiology*, *92*(6), 2245–2255.

Levine, J. A., Lanningham-Foster, L. M., McCrady, S. K., Krizan, A. C., Olson, L. R., Kane, P. H., et al. (2005). Interindividual variation in posture allocation: Possible role in human obesity. *Science*, *307*, 584–586.

Lisse, T. S., Thiele, F., Fuchs, H., Hans, W., Przemeck, G. K., Abe, K., et al. (2008). ER stress-mediated apoptosis in a new mouse model of osteogenesis imperfecta. *PLoS Genetics*, *4*(2), e7.

Lochmüller, E. M., Jung, V., Weusten, A., Wehr, U., Wolf, E., & Eckstein, F. (2001). Precision of high-resolution dual energy X-ray absorptiometry of bone mineral status and body composition in small animal models. *European Cells & Materials*, *1*, 43–51.

Maier, K. L., Alessandrini, F., Beck-Speier, I., Hofer, T. P., Diabaté, S., Bitterle, E., et al. (2008a). Health effects of ambient particulate matter – biological mechanisms and inflammatory responses to in vitro and in vivo particle exposures. *Inhalation Toxicology*, *20*(3), 319–337.

Maier, H., Lengger, C., Simic, B., Fuchs, H., Gailus-Durner, V., & Hrabé de Angelis, M. (2008b). MausDB: An open source application for phenotype data and mouse colony management in large-scale mouse phenotyping projects. *BMC Bioinformatics*, *9*, 169.

Mallon, A. M., Blake, A., & Hancock, J. M. (2008). EuroPhenome and EMPReSS: Online mouse phenotyping resource. *Nucleic Acids Research*, *36*(Database issue), D715–D718.

Mariani, F. V., & Martin, G. R. (2003). Deciphering skeletal patterning: Clues from the limb. *Nature*, *423*(6937), 319–325.

Masliah, E., Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., et al. (2000). Dopaminergic loss and inclusion body formation in alpha-synuclein mice: Implications for neurodegenerative disorders. *Science*, *287*, 1269.

McLean, W., & Olsen, B. R. (2001). Mouse models of abnormal skeletal development and homeostasis. *Trends in Genetics*, *10*, S38–S43.

Meyer, C. W., Klingenspor, M., Rozman, J., & Heldmaier, G. (2004). Gene or size: Metabolic rate and body temperature in obese growth hormone-deficient dwarf mice. *Obesity Research*, *12*(9), 1509–1518.

Miczek, K. A. (1999). Aggressive and social stress responses in genetically modified mice: From horizontal to vertical strategy. *Psychopharmacology (Berlin)*, *147*, 17–19.

Mindnich, R., & Adamski, J. (2007). Functional aspects of 17beta-hydroxysteroid dehydrogenase 1 determined by comparison to a closely related retinol dehydrogenase. *The Journal of Steroid Biochemistry and Molecular Biology*, *104*(3–5), 334–339.

Möller, G., & Adamski, J. (2006). Multifunctionality of human 17beta-hydroxysteroid dehydrogenases. *Molecular and Cellular Endocrinology*, *248*(1–2), 47–55.

Morino, K., Neschen, S., Bilz, S., Sono, S., & Tsirigotis, D. (2008). Muscle specific IRS-1 Ser → Ala transgenic mice are protected from fat-induced insulin resistance in skeletal muscle. *Diabetes*, *57*, 2644–2651.

Moßbrugger, L., Hölzlwimmer, G., Calzada-Wack, J., & Quintanilla-Martinez, L. (2007). Standardized morphological phenotyping of mouse models of human diseases within the German Mouse Clinic. *Verhandlungen der Deutschen Gesellschaft für Pathologie*, *91*, 98–103.

Nadeau, J. H., Balling, R., Barsh, G., Beier, D., Brown, S. D., Bucan, M., et al. (2001). Sequence interpretation. Functional annotation of mouse genome sequences. *Science*, *291*(5507), 1251–1255.

Neschen, S., Katterle, Y., Augustin, R., & Scherneck, S. (2008). Uncoupling protein 1 expression in murine skeletal muscles increases AMPK activation, glucose turnover and insulin sensitivity in vivo. *Physiological Genomics*, *33*, 333–340.

Neschen, S., Morino, K., Dong, J., Wang-Fischer, Y., & Cline, G. W. (2007). n-3 fatty acids preserve insulin sensitivity in vivo in a peroxisome proliferator-activated receptor-alpha-dependent manner. *Diabetes*, *56*, 1034–1041.

Neschen, S., Morino, K., Hammond, L. E., Zhang, D., & Liu, Z. X. (2005). Prevention of hepatic steatosis and hepatic insulin resistance in mitochondrial acyl-CoA:Glycerol-sn-3-phosphate acyltransferase 1 knock out mice. *Cell Metabolism*, *2*, 55–65.

Nikitin, A. Y., Alcaraz, A., Anver, M. R., Bronson, R. T., Cardiff, R. D., Dixon, D., et al. (2004). Classification of proliferative pulmonary lesions of the mouse: Recommendations of the mouse models of human cancers consortium. *Cancer Reserach*, *64*, 2307–2316.

Nolan, P. M., Peters, J., Vizor, L., Strivens, M., Washbourne, R., Hough, T., et al. (2000). Implementation of a large-scale ENU mutagenesis program: Towards increasing the mouse mutant resource. *Mammalian Genome*, *11*, 500–506.

Paigen, K. (2003a). One hundred years of mouse genetics: An intellectual history. I. The classical period (1902–1980). *Genetics*, *163*(1), 1–7.

Paigen, K. (2003b). One hundred years of mouse genetics: An intellectual history. II. The molecular revolution (1981–2002). *Genetics*, *163*(4), 1227–1235.

Pawlak, C. R., Sanchis-Segura, C., Soewarto, D., Wagner, S., Hrabě de Angelis, M., & Spanagel, R. (2008). A phenotype-driven ENU mutagenesis screen for the identification of dominant mutations involved in alcohol consumption. *Mammalian Genome*, *19*(2), 77–84.

Peters, L. L., Robledo, R. F., Bult, C. J., Churchill, G. A., Paigen, B. J., & Svenson, K. L. (2007). The mouse as a model for human biology: A resource guide for complex trait analysis. *Nature Reviews Genetics*, *8*(1), 58–69.

Petkova, S. B., Yuan, R., Tsaih, S. W., Schott, W., Roopenian, D. C., & Paigen, B. (2008). Ge-netic influence on immune phenotype revealed strain-specific variations on pe-ripheral blood lineages. *Physiological Genomics*, *34*(3), 304–314.

Prehn, C., Ströhle, F., Haller, F., Keller, B., Hrabě de Angelis, M., Adamski, J., et al. (2007). A Comparison Of Methods For Assays Of Steroidogenic Enzymes: New GC/MS Versus HPLC And TLC. In H. Weiner, B. Plapp, R. Lindhal, & E. Maser, (eds), *Enzymology and molecular biology of carbonyl metabolism* (Vol. 13. pp. 277–283). West Lafayette, IN: Purdue University Press.

Ralston, S. H., & de Crombrugghe, B. (2006). Genetic regulation of bone mass and susceptibility to osteoporosis. *Genes & Development*, *20*(18), 2492–2506.

Rathkolb, B., Decker, T., Fuchs, E., Soewarto, D., Fella, C., Heffner, S., et al. (2000). The clinical-chemical screen in the Munich ENU Mouse Mutagenesis Project: Screening for clinically relevant phenotypes. *Mammalian Genome*, *11*(7), 543–546.

Rauch, F., & Glorieux, F. H. (2004). Osteogenesis imperfecta. *Lancet*, *363*(9418), 1377–1385.

Reaven, G. M. (1995). Pathophysiology of insulin resistance in human disease. *Physiological Reviews*, *75*(3), 473–486.

Roederer, M. (2001). Spectral compensation for flow cytometry: Visulatization artifacts, limitations, and caveats. *Cytometry*, *45*, 194–205.

Roederer, M., Nozzi, J. L., & Nason, M. C. (2011). SPICE: Exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry Part A, 79A*: 167–174.

Rosemann, M., Ivashkevich, A., Favor, J., Dalke, C., Hölter, S. M., Becker, L., et al. (2010). Microphthalmia, parkinsonism, and enhanced nociception in Pitx3 (416insG) mice. *Mammalian Genome*, *21*, 13–27.

Rosen, C. J., Beamer, W. G., & Donahue, L. R. (2001). Defining the genetics of osteoporosis: Using the mouse to understand man. *Osteoporosis International*, *12*(10), 803–810.

Roskam, S., Neff, F., Schwarting, R., Bacher, M., & Dodel, R. (2010). APP transgenic mice: The effect of active and passive immunotherapy in cognitive tasks. *Neuroscience and Biobehavioral Reviews*, *34*(4), 487–499.

Rothwell, N. J., & Stock, M. J. (1981). Regulation of energy balance. *The Annual Review of Nutrition*, *1*, 235–256.

Schmidt, C., Priemel, M., Kohler, T., Weusten, A., Muller, R., Amling, M., et al. (2003). Precision and accuracy of peripheral quantitative computed tomography (pQCT) in the mouse skeleton compared with histology and microcomputed tomography (microCT). *Journal of Bone and Mineral Research*, *18*(8), 1486–1496.

Schneider, I., Tirsch, W. S., Faus-Kessler, T., Becker, L., Kling, E., Busse, R. L., et al. (2006). Systematic, standardized and comprehensive neurological phenotyping of inbred mice strains in the German Mouse Clinic. *Journal of Neuroscience Methods*, *157*(1), 82–90.

Shappell, S. B., Thomas, G. V., Roberts, R. L., Herbert, R., Ittmann, M. M., Rubin, M. A., et al. (2004). Prostate pathology of genetically engineered mice: Definitions and classification. The consensus report from the Bar Harbor meeting of the Mouse Models of Human Cancer Consortium Prostate Pathology Committee. *Cancer Research*, *64*, 2270–2305.

Soewarto, D., Klaften, M., & Rubio-Aliaga, I. (2009). Features and strategies of ENU mouse mutagenesis. *Current Pharmaceutical Biotechnology*, *10*(2), 198–213.

Solberg, L. C., Valdar, W., Gauguier, D., Nunez, G., Taylor, A., Burnett, S., et al. (2006). A protocol for high-throughput phenotyping, suitable for quantitative trait analysis in mice. *Mammalian Genome*, *17*(2), 129–146.

Somel, M., Creely, H., Franz, H., Mueller, U., Lachmann, M., Khaitovich, P., et al. (2008). Human and chimpanzee gene expression differences replicated in mice fed different diets. *PLoS One*, *3*(1), e1504.

Sorenson, J. A., Duke, P. R., & Smith, S. W. (1989). Simulation studies of dual-energy X-ray absorptiometry. *Medical physics*, *16*, 75–80.

Speakman, J., Hambly, C., Mitchell, S., & Król, E. (2008). The contribution of animal models to the study of obesity. *Laboratory Animal*, *42*(4), 413–432.

Sugiyama, F. (1997). Development of genetically engineered mice with hypertension and hypotension. *Experimental Animals*, *46*, 171–182.

Tomlinson, J. W., & Stewart, P. M. (2007). Modulation of glucocorticoid action and the treatment of type-2 diabetes. *Best Practice & Research Clinical Endocrinology & Metabolism*, *21*(4), 607–619.

Uttara, B., Singh, A. V., Zamboni, P., & Mahajan, R. T. (2009). Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options. *Current Neuropharmacology*, *7*(1), 65–74.

van Buerck, L., Blutke, A., Kautz, S., Rathkolb, B., Klaften, M., Wagner, S., et al. (2010). Phenotypic and pathomorphological characteristics of a novel mutant mouse model for maturity-onset diabetes of the young type 2 (MODY 2). *American Journal of Physiology – Endocrinology and Metabolism*, *298*(3), E512–E523.

Villareal, O., Villarreal, J. J., & Domingo, J. A. et al. (1999). Progressive eosinophilia and elevated IgE in enterobiasis. *Allergy*, *54*, 646–648.

Wakana, S., Suzuki, T., Furuse, T., Kobayashi, K., Miura, I., Kaneda, H., et al. (2009). Introduction to the Japan Mouse Clinic at the RIKEN BioResource Center. *Experimental Animals*, *58*(5), 443–450.

Waldman, T. A., Polmar, S. H., Balestra, S. T., Jost, M. C., Bruce, R. M., & Terry, W. D. et al. (1972). Immunoglobulin E in immunologic deficiency diseases, II, Serum IgE concentration of patients with acquired hypogammaglobulinemia, thymoma and hypogammaglobulinemia, myotonic dystrophy, intestinal lympangiectasia and wiskott-aldrich syndrome. *The Journal of Immunology*, *109*, 304–310.

Wang-Sattler, R., Yu, Y., Mittelstrass, K., Lattka, E., Altmaier, E., Gieger, C., et al. (2008). Metabolic profiling reveals distinct variations linked to nicotine consumption in humans–first results from the KORA study. *PLoS ONE*, *3*(12), e3863.

Weiss, W. A., Israel, M., Cobbs, C., Holland, E., James, C. D., Louis, D. N., et al. (2002). Neuropathology of genetically engineered mice: Consensus report and recommendations from an international forum. *Oncogene*, *21*, 7453–7463.

Wu, H., Liu, X., & Jaenisch, R. (1994). Double replacement: Strategy for efficient introduction of subtle mutations into the murine Col1a-1 gene by homologous recombination in embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, *91*, 2819–2823.

Ying, S. Y., & Lin, S. L. (2009). Intron-mediated RNA interference and microRNA biogenesis. *Methods in Molecular Biology*, *487*, 387–413.

Zeyda, M., & Stulnig, T. M. (2009). Obesity, inflammation, and insulin resistance – A mini-review. *Gerontology*, *55*, 379–386.

Chapter 3
Nature and Nurture: Impacts on Mouse Phenotypes and Translational Research

Cory Brayton

Abstract While genome projects continue to provide crucial information about the structure of genomes and their genes, functional genomics (or functional genetics) initiatives strive to understand the function of genomes and their genes. Identification of a protein gene product and determination of its function as a receptor, ligand or enzyme is a proximal goal. Understanding the role of the gene and its product, in the context of a living organism, in its environment is the ultimate goal of functional genomics initiatives. Thus, functional genomics efforts in genetically engineered animals aim to produce and characterize phenotype(s) that clearly result from the intended genetic manipulation(s) and help to elucidate gene function(s). However, phenotypes reflect genetic influences other than the intended genetic manipulations, as well as experiential and environmental influences including infectious agents. Potential impacts of extra-experimental variables must be considered when interpreting phenotype data.

3.1 Introduction

Although the concepts had been expressed previously by Mendel, Darwin and others, the terms "gene", "genotype" and "phenotype" were introduced by the Danish botanist Wilhelm Johannsen around 1909 (Johannsen, 1911; Winge, 1958).

Phenotype comes from Φαινό- (phainen, display or show, as in phenomenon) and τύπος (typos or type), loosely translating to "form as shown". Phenotype refers to any trait, i.e. detectable or measurable feature of an organism, ranging from body and organ sizes, hair and eye colors, to blood cell counts, enzyme activities, molecular characterizations, and any observable or measurable signs of illness. Phenotypes include "normal" findings in healthy control animals, as well as "abnormal" findings

C. Brayton (✉)
Molecular and Comparative Pathobiology, School of Medicine, Johns Hopkins University, Baltimore, MD, USA
e-mail: cbrayton@jhmi.edu

C. Brakebusch, T. Pihlajaniemi (eds.), *Mouse as a Model Organism*,
DOI 10.1007/978-94-007-0750-4_3,

in ill or manipulated animals. The earliest pubmed citation for the search term "phenotype" is 1912 (Shull, 1912). Accessed March 12, 2010.

Phenotyping can be considered to comprise any/all evaluations of phenotypes, regardless of the species or the proximate cause or contributors to the phenotypes. Ideal phenotyping evaluations are minimally invasive, inexpensive, high throughput, quantifiable, and easily reproducible with high yield of discriminating results that clearly distinguish deviations from the normal or control phenotypes. Most real phenotyping evaluations are deficient in at least one of these qualities. The earliest citation in Pubmed (searched Feb 12, 2010) for the term "phenotyping" is 1965 (Fredrickson & Lees, 1965).

Phenotyping strategies for Genetically Engineered Mice (GEM) or other Genetically Modified Organisms (GMO) include combinations of in vivo evaluations, imaging strategies, molecular evaluations (microarray, proteomics, metabolomics etc), and clinical and anatomic pathology. Strategies necessarily reflect local resources, expertise and research interests.

The European Mouse Phenotyping Resource of Standardized Screens (EMPRESS http://empress.har.mrc.ac.uk/) and the Mouse Phenome Database based at the Jackson Laboratory (MPD http://phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home) are online resources that continue to grow to provide more phenotype data and phenotyping protocol details.

GEM (genetically engineered mice) or other **GMO** (genetically modified organisms) often are valuable or unique. Their phenotypes manifest at different ages, change over time, may be induced to appear or disappear, or expected phenotypes may not be evident. In vivo evaluations preserve GEM for breeding and further testing, and for retesting in longitudinal studies (as they develop and age). In vivo phenotyping evaluations range from relatively inexpensive observations and measurements (e.g. eye and coat color, subjective evaluation of morphology/dysmorphology, body condition, activity, and behavior; body weights for growth curves), to behavioral, metabolic, physiologic, or imaging assessments that require substantial equipment investments, to diet manipulations, and invasive interventions that require substantial expertise.

Pathology remains a critical tool in disease diagnosis and characterization, and is mandated in assessment and development of drugs and devices to treat human diseases, which is the ultimate aim of much preclinical research. New virtual (digital) microscopy technologies can facilitate and expedite consulting, quantitative analysis, electronic archiving and publication quality image capture. But pathology processing and assessment usually is terminal for the subject, and requires substantial investments of time and expertise. Nonetheless, strategic planned use of pathology in phenotyping GEM, and in other areas of biomedical research, can provide crucial characterization of similarities and differences between a potential model and condition it is hypothesized to model. Strategic use of pathology to analyze unexpected phenotypes can help to identify problems and causes early, to minimize loss of animals, money and time, and to prevent serious compromise of a project (Barthold, 2002; Brayton, Justice, and Montgomery, 2001; Cardiff, 2009; Cardiff et al., 2004).

Examples of neurobehavioral and of cancer phenotypes in mice, are discussed below. Some relevant pathology findings, and genetic and environmental influences that can contribute to them illustrate the potential contributions of nature, nurture and pathology to phenotyping and to translational research. Phenotypes that are partially or largely related to nature and/or nurture, may be useful or "good" for the study, e.g. heritable disease susceptibility or resistance, or an obvious disease phenotype that is not evident in "cleaner" mice. But phenotypes partially or largely related to nature and/or nurture can also complicate or confound a study, e.g. infection related morbidity or mortality that resembles an expected phenotype or shortens a study.

3.2 Condition: Retinal Degeneration

Clinical/Behavioral Phenotypes: Poor performance in tests that require vision (Brown and Wong, 2007)

Gross Pathology Findings (dysmorphology): Not evident without special equipment or techniques (e.g. fundic exam or histopathology).

Histopathology Findings: No photoreceptors in retina, complete diffuse bilateral, is characteristic of homozygosity for *Pde6b*^{rd1} *(rd1)*, called "rodless retina". Other types of retinal degeneration in mice may have dysplastic changes such as folds or rosettes (Chang et al., 2002).

Nature Influences: Phosphodiesterase 6B (*Pde6b*), cGMP, rod receptor, beta polypeptide locus, is on chromosome 5. Mice Homozygous for its recessive *rd1* allele (*Pde6b*^{rd1}) lose all rod cells by 35 days of age. Cones degenerate slower, and some light sensitivity may remain (Jimenez et al., 1996; Jones & Marc, 2005; Pittler et al., 1993; Zeiss, Neal, & Johnson, 2004). The genotype and phenotype are expected in C3H, CBA, FVB/N, SJL/J, MOLF/EiJ strains and some Swiss stocks (Clapcote et al., 2005; Serfilippi et al., 2004). Allelic mutations result in similar or milder phenotypes. A rodless retina phenotype was described by Keeler in 1924 in "white" (Swiss?) mice (Keeler, 1924). Bruckner Identified a similar phenotype in the 1950's (Pittler et al., 1993). Various other mutations or genotypes can contribute to heritable retinal dysplasia or degeneration in mice but *rd1* probably is the most common and easily recognized in common inbred strains (Chang et al., 2002). Albino mammals, including mice, lack pigment in their retinal pigment epithelium, and may have other retina or neuron defects that contribute to elevated visual thresholds, poor visual acuity and or poor depth perception (Balkema & Drager, 1991; Rachel et al., 2002; Wong & Brown, 2006).

Nurture Influences: Excessive light is a recognized cause of retinal degeneration. Albino mice in open top cages near ceiling light fixtures were especially likely to develop changes (Greenman et al., 1982). In covered, or closed and ventilated caging that is more common now, this seems less likely to be a problem.

3.3 Condition: Microphthalmia

Clinical/Behavioral Phenotypes: Small eyes, or not evident; poor performance in tests that require vision.

Gross Pathology Findings (dysmorphology): Small eyes. Congenital microphthalmia should be evident by the time of weaning, as obviously small or apparently absent eye(s).

Histopathology Findings: An array of abnormalities in anterior, posterior chambers, lens and retina, usually with marked disorganization, loss or apparent failure to develop fully. Complete anophthalmia is rare. Usually some eye structures can be identified (Smith, Roderick & Sundberg, 1994). Differential diagnosis: phthisis bulbi, likely related to trauma.

Nature Influences: Microphthalmia is reported to be most common in C57BL strains, most commonly in females in the right eye, with an incidence up to 12% (Smith et al., 1994). Multiple genes have been identified, e.g. *Mitf* (microphthalmia associated transcription factor). Some variation in eye size and other anatomic features is expected among strains (Puk et al., 2006).

Nurture: Phthisis bulbi typically related to ocular trauma results in a small eye that could resemble microphthalmia on clinical and gross examination. A history of retroorbital bleeding would be highly suggestive.

3.4 Condition: Glaucoma

Clinical/Behavioral Phenotype: Poor performance in tests that require vision.

Gross Pathology Findings (dysmorphology): In non dilated eyes, loss of iris or lacy iris remnants, consistent with iris atrophy, might be identified without magnification, but magnification or slit lamp microscopy will facilitate diagnosis.

Histopathology Findings: Iris atrophy and pigment dispersion, later onset retinal degeneration and photoreceptor loss.

Nature Influences: Glaucoma is expected in DBA/2 mice, and attributed to *Tyrp1*^{isa} (iris stromal atrophy mutation in tyrosinase-related protein 1 gene), and to *Gpnmb*^{R150X} (iris pigment dispersion mutation in glycoprotein nmb gene). Vision loss is expected between 6 and 12 months of age in DBA/2 J mice (Anderson et al., 2008; Chang et al., 1999; John et al., 1998; Sheldon et al., 1995; Wong & Brown, 2007).

3.5 Condition: Cataracts (and Corneal Opacities)

Clinical/Behavioral Phenotype: Lenticular opacities, or corneal opacities, possibly poor performance in tests that require vision.

Gross Pathology Findings (dysmorphology): Lenticular opacities (white areas **in** eyes) should be differentiated from corneal opacities (opaque,

usually white areas **on** eyes' corneal surface). Mild cataractous changes can be identified by slit lamp microscopy or histology before they are evidently clinically or grossly (Hubert, Gerin, & Durand-Cavagna, 1999). Mild subcapsular and cortical cataracts may be identified in old mice (28–31 m) (Pendergrass et al., 2005).

Histopathology Findings: Histopathology of lenticular cataractous changes varies from mild globular changes, vacuolation of epithelial cells or few large "bladder" cells in nuclear, anterior, posterior portions of lens to severely disorganized, disrupted fibers, displacement of nucleus, or more severe changes, such as synechiae or ruptured capsule. Some references reserve the term cataract for irreversible lenticular opacities present at birth (Taradach & Greaves, 1984).

Histopathology of corneal opacities (also called corneal dystrophy) usually includes mineralization, inflammation and neovascularization and or edema in the cornea (Van Winkle & Balk, 1986; Yamate et al., 1987).

Nature Influences: Many cataract associated genes have been identified, with many discovered as autosomal dominant or recessive traits (phenotypes) in mutagenesis studies. Almost 200 cataract associated loci or alleles are identified in MGI (Mouse Genome Informatics database at http://www.informatics.jax.org/ accessed March 12, 2010). The Emory cataract mouse (derived from CFW stock) carries autosomal dominant trait for late onset cataract, but many of the mouse cataract models are of congenital cataracts (Graw, 2009).

BALB/c, C3H, DBA/2, and MRL/Mp mice, are be more likely to develop corneal opacities than other common strains (Taradach & Greaves, 1984; Van Winkle & Balk, 1986; Verhagen et al., 1995; Yamate et al., 1987).

Nurture Influences: Some reversible lenticular opacities (cataracts) or corneal opacities can be induced by anesthetics and other chemicals or conditions (Calderone, Grimes, & Shalev, 1986; Fraunfelder & Burns, 1970; Ridder, Nusinowitz & Heckenlively, 2002; Vieira et al., 2009). Mutagens such as ENU and irradiation can cause mutations that result in cataracts (Jablonski et al., 2005; West & Fisher, 1986). Streptzootocin induced mouse diabetes models may develop cataracts (Hegde & Varma, 2005). Ultraviolet irradiation can cause cataracts and is used experimentally in mice (Meyer et al., 2005).

Corneal opacities are usually attributed to trauma or other causes of corneal damage in susceptible mouse strains (Taradach & Greaves, 1984; Van Winkle & Balk, 1986).

3.6 Condition: Presbyacusis (Age Related Hearing Loss)

Clinical/Behavioral Phenotype: Poor performance in tests that require hearing; increased ABR thresholds.

Gross Pathology Findings (dysmorphology): None expected

Histopathology Findings: Degeneration in organ of Corti; necrosis, degeneration and loss of hair cells and/or spiral ganglion cells (Keithley et al., 2004; Willott & Erway, 1998).

Nature Influences: Many inbred strains have elevated ABR thresholds by 3mo: 129/J, 129/ReJ, 129/SvJ, A/J, C57BL/6 J, C57BLKS/J, C57L/J, DBA/2 J, NOD/LtJ, NOR/LtJ, SKH2/J, etc. C3H and CBA mice are expected to retain ABR thresholds, with Longer lived CBA strains frequently used as controls in hearing studies. Ahl (*Chd23*^{ahl} in cadherin 23 locus on Chromosome 10) is a major contributor to presbyacusis in C57BL/6 J, A/J, BALB/cByJ, BUB/BnJ, C57BR/cdJ, DBA/2 J, NOD/LtJ, SKH2/J, but other genetic contributors have been identified. DBA/2 mice lose their organ of Corti by 6 months of age and also may have vestibular symptoms (circling) (Hultcrantz & Spangberg, 1997; Willott et al., 2003).

Nurture Influences: Noise (Davis et al., 2001; Turner et al., 2005; Willott, Bross, & McFadden, 1994); infectious agents that cause otitis (Mitchell et al., 1997); diet (Willott et al., 1995); ototoxic compounds or drugs e.g. Gentamycin, Kanamycin, Cisplatin (Poirrier et al.).

3.7 Condition: Otitis Media (Inflammation in Middle Ear)

Clinical/Behavioral Phenotype: Possibly poor performance in tests that require hearing; increased ABR Thresholds (McGinn, Bean-Knudsen, & Ermel, 1992; Mitchell et al., 1997); inflammatory (reactive) leukocytosis and cytokine responses, possibly fever, reduced activity or signs of illness.

Gross Pathology Findings (dysmorphology): Not identified, or exudate (pus) in bullae.

Histopathology Findings: Inflammation, exudates in middle ear (bulla), readily identified in decalcified head sections. Chronic otitis findings can include acicular clefts and granulomatous inflammation, as in cholesteatoma in gerbils or humans with chronic otitis. Foreign material such as hair or plant debris, indicates rupture of the tympanum (ear drum).

Nature Influences: Immune deficiency or other genetically determined susceptibility to infectious agents, such as Tlr4 deficiency, due to homozygosity for *Tlr4*^{lps} in C3H/HeJ mice (Bleich et al., 2008).

Nurture Influences: Infectious agents: Gram positive agents including *Streptococcus spp* (Prince et al., 2001); Gram negative opportunists such as *Klebsiella oxytoca (*Macarthur et al., 2008), *Pasteurella pneumotropica (*Goelz et al., 1996).

3.8 Condition: Otitis Interna (Inflammation in Inner Ear)

Clinical/Behavioral Phenotype(s): Possibly poor performance in tests that require hearing or assess neuromuscular function; possibly head tilt, or obviously circling mice, rolling, or spinning mice; increased ABR Thresholds;

possibly inflammatory (reactive) leukocytosis and cytokine responses, possibly fever, reduced activity.

Gross Pathology Findings (dysmorphology): usually none.

Histopathology Findings: Inflammation or exudates in inner ear (labyrinthitis, otitis interna) (Kohn & MacKenzie, 1980) are readily identified in decalcified head sections.

Nature Influences: Immune deficiency or other genetically determined susceptibility to infectious agents. DBA/2, Ames waltzer and other mouse mutants have genetically determined vestibular abnormalities (Hultcrantz & Spangberg, 1997; Jones et al., 2006; Raphael et al., 2001). Otitis should be considered as a cause or contributor to vestibular phenotypes.

Nurture Influences: Consider similar infectious agents as in otitis media (Dietrich, Khaschabi, & Albini, 1996; Kohn & MacKenzie, 1980); possibly viral infections (Davis, 1990; Shimokata et al., 1977).

3.9 Condition: Arteritis, Polyarteritis

Clinical/Behavioral Phenotype(s): None, possible loss of condition, signs of illness, or vestibular signs such as rolling, spinning, circling, head tilt, or poor performance in tests that require hearing or assess neuromuscular function.

Gross Pathology Findings (dysmorphology): None expected.

Histopathology Findings: Arteritis/polyarteritis can be identified in many tissues, including in head near cochlea, vestibular ganglia or nerves. Arteritis in or near the head may be an incidental finding in the absence of reported clinical signs. Arteritis is likely at other sites, such as coronary arteries, mesentery, pancreas stomach, testis.

Nature Influences: Arteritis/polyarteritis can be a mild incidental finding, not associated with clinical signs, in chronic studies on various strains. It has been associated with autoimmunity, e.g. Mrl, Mrl ^{lpr} mice (Hewicker & Trautwein, 1987; Nose et al., 2000; Qu et al., 2000),implicated in vestibular syndrome, or death (with severe cardiac involvement) (Hewicker & Trautwein, 1987; Maita et al., 1988; Qu et al., 2000). Arteritis has been associated with hypertension in mice and rats (Mullink & Haneveld, 1979).

Nurture Influences: Infectious causes of this sporadic condition seem likely, and the incidence seems to be lower in more recent studies with lower pathogen prevalence, but an infectious cause has not been identified definitively. Experimental infections with MHV3 can cause thrombotic vasculitis in susceptible mice (Tardieu et al., 1982).

3.10 Condition: Brain (CNS), Infarction

Clinical/Behavioral Phenotype(s): Possibly acute onset rolling, spinning, circling.

Gross Pathology Findings (dysmorphology): Malacia (softening and altered color) in brain stem may be identified when fixed brain or decalcified head is sectioned.

Histopathology Findings: Unilateral brainstem lesions of rarefaction and necrosis of brain near vestibular nuclei resembles Wallenberg's Lateral Medullary Syndrome in humans.

Nature Influences: Unilateral brainstem infarction reported in unmanipulated female Swiss mice less than 1 yr old (Southard & Brayton). Variations in cerebral vascularization have been identified within and between mouse strains. C57BL/6 and CD-1 mice may have a high incidence of incomplete or incompetent posterior communicating artery perfusion, and are used to model global or forebrain ischemia by carotid ligation and other techniques (Beckmann, 2000; Yang et al., 1997; Yonekura et al., 2004).

Nurture Influences: Undetermined.

3.11 Condition: Weakness, Wasting

Clinical/Behavioral Phenotype(s): Weak wasted or ill mice may not move much in any test; may perform poorly on rotarod or in swim test (Bailey, Rustay, & Crawley, 2006).

Gross Pathology Findings (dysmorphology): Wasted, possibly hunched mice with reduced muscle mass, may not have other obvious gross lesions. Neoplasia or other contributors to clinical deterioration may be identified.

Histopathology Findings: Small myofibers, muscle atrophy, "hypercellular" fat due to reduced adipocyte size are non specific findings. Amyloidosis, arteritis, acidophilic macrophage pneumonia, glomerulonephritis, enterocolitis, or other contributors to illness or wasting phenotypes may be identified.

Nature Influences: Various genetic susceptibilities to systemic disease such as amyloidosis, or to infections.

Nature Influences: Various. Consider infectious agents, especially in conventional housing conditions.

3.12 Condition: Muscular Dystrophy

Clinical/Behavioral Phenotype(s): May grasp limbs instead of climbing tail in tail hang; may swim abnormally or perform poorly on rotarod; possible cardiac dysfunction.

Gross Pathology Findings (dysmorphology): Depending on type, degree and severity, decreased or increased muscle mass, or cardiomegaly may be identified.

Histopathology Findings: Depending on the type or cause, there may be variation in fiber size, varying degrees of myofiber degeneration, myocytolysis,

loss of striation, internal nuclei; +/– inflammation; regeneration with internal nuclei in tandem array or replacement fibrosis with fibrocollagen (Vainzof et al., 2008).

Nature Influences (Vainzof et al., 2008): Dysferlin mutations in A/J and SJL/J mice (Ho et al., 2004; Wenzel et al., 2007); spontaneous mutations in Lama2 (merosin or laminin, alpha2) in 129/Re mice (Jasmin and Bajusz, 1962; Zdanowicz et al., 1995), or in Dystrophin in (Mdx) mice (Lefaucheur, Pastoret, & Sebille, 1995).

Nurture Influences: Diet and drug interventions may modulate some myopathy and muscular dystrophy phenotypes (Girgenrath et al., 2009; Zdanowicz et al., 1995).

3.13 Condition: CNS, Demyelination

Clinical/Behavioral Phenotype(s): Paresis (weakness), paralysis

Gross Pathology Findings (dysmorphology): Depending on chronicity and severity, muscle wasting of affected areas may be identified.

Histopathology Findings: Swollen myelin sheaths, possibly swollen axons (spheroids), possible inflammation depending on cause. Muscle atrophy secondary to denervation may be identified. With mouse hepatitis virus (MHV) acute lesions can include encephalitis with microglial nodules (also liver necrosis and inflammation) (Azoulay-Cayla et al., 2001; Coley et al., 2005; Dandekar & Perlman, 2002; Deb et al., 2009; Kumar et al., 2004; Lavi et al., 1986; Tsunoda et al., 1996; Turrin, 2008; Woyciechowska et al., 1984).

Nature Influences: Susceptibility to infection or disease (Azoulay-Cayla et al., 2001; Coley et al., 2005; Dandekar & Perlman, 2002; Deb et al., 2009; Kumar et al., 2004; Lavi et al., 1986; Tsunoda et al., 1996; Turrin, 2008; Woyciechowska et al., 1984).

Nurture Influences: Mouse Hepatitis virus (MHV) (Coley et al., 2005; Dandekar & Perlman, 2002; Lavi et al., 1986; Woyciechowska et al., 1984) and Theiler's Mouse Encephalitis Virus (TMEV) (Azoulay-Cayla et al., 2001; Deb et al., 2009; Kumar et al., 2004; Tsunoda et al., 1996; Turrin, 2008) have been used to model human demyelinating diseases (e.g. multiple sclerosis, and polio) in susceptible mouse strains.

3.14 Condition: Hydrocephalus

Clinical/Behavioral Phenotype(s): Subclinical to moribund depending on age, progression rate, and severity. Young animals that develop dilated ventricles before cranial sutures close can have obviously "domed" expanded head contour.

Gross Pathology Findings (dysmorphology): Domed head in young animals; possible coning of cerebellum through foramen magnum. Subgross sectioned

brain, or sectioned decalcified heads have variably dilated ventricles and peripherally compressed brain, or collapsed, depressed cortex attributed to loss of pressure during dissection.

Histopathology Findings: Ventricle dilatation (or collapse) consistent with subgross, with variable degrees of brain compression, atrophy.

Nature Influences: Hydrocephalus can be common in C57BL colonies, with domed head evident at weaning (Carton et al., 1956). C57BL/6 have relatively large ventricles (Zygourakis & Rosen, 2003). Later onset hydrocephalus can occur in MRL mice (Denenberg et al., 1992). Domed head is not evident if the condition develops after cranial sutures close, but hydrocephalus usually can be diagnosed during sectioning of brain or decalcified head.

Nurture Influences: Various experimental viral infections can cause hydrocephalus, and/or cerebellar hypoplasia or dysplasia (Hausler et al., 2005; Holtz, Borman and Li, 1966; Kristensson et al., 1984; Lagace-Simard, Descoteaux, & Lussier, 1980; Margolis & Kilham, 1969; Tardieu et al., 1982; Tsunoda et al., 1997). There is not much information regarding hydrocephalus in natural infections.

3.15 Condition: CNS, Hypocallosity, Acallosity

Clinical/Behavioral Phenotype(s): Possibly altered sociability and other behaviors (Balogh et al., 1999; Brodkin, 2007; Filgueiras & Manhães, 2004).

Gross Pathology Findings (Dysmorphology): Absence of corpus callosum (acallosity) can be identified by examination of sectioned brain or decalcified head.

Histopathology Findings: Acallosity is identified easily. Hypocallosity is assessed by morphometry or imaging techniques.

Nature Influences: Hypocallosity and acallosity are reported in 129 and BALB/c (Balogh et al., 1999; Brodkin, 2007; Livy & Wahlsten, 1991; Wahlsten, Bishop, & Ozaki, 2006) with incidences up to 70%. BTBR T+ tf/J mice have 100% absence of corpus callosum and a severely reduced hippocampal commissure (Wahlsten, Metten, & Crabbe, 2003).

Nurture Influences: Incidence of acallosity has been reported to vary with identical strains reared in different environments, but specific factors are unclear (Wahlsten, 1982).

3.16 Condition: Seizures

Clinical/Behavioral Phenotype(s): facial grimace, chewing, ptyalism, matted fur, convulsions, or found dead.

Gross Pathology Findings (dysmorphology): Non specific.

Histopathology Findings: Minimal to striking neuron necrosis in hippocampus, cortex, thalamus, amygdala, astrocytosis can be a feature, depending on the strain and inciting stimuli (Drage, Holmes & Seyfried, 2002; Goelz et al., 1998; McLin & Steward, 2006).

Nature Influences: DBA/2 mice are susceptible to noise induced seizures that may be fatal, until they become deaf with significant inner ear pathology. Some DBA/2 mice develop vestibular symptoms (circling) (Bloom & Hultcrantz, 1994; Jones et al., 2006). FVB/N mice are susceptible to spontaneous and induced seizures (Goelz et al., 1998; Mohajeri et al., 2004).

Nurture Influences: Noise, etc stimuli; Kainic acid, pilocarpine and other epileptogenic drugs or stimuli (Engstrom & Woodbury, 1988; Golden et al., 2001; Hom, Leppik, & Rask, 1993; McLin & Steward, 2006).

3.17 Condition: Neoplasia affecting CNS, vestibular system, special senses or other systems

Clinical/Behavioral Phenotype(s): Weak or moribund phenotypes; paresis paralysis when spine is involved; possible rolling, spinning, circling, head tilt when vestibular system is involved; altered responses in tests that require vision or hearing if those systems are involved.

Gross Pathology Findings (dysmorphology): Tumor/mass involving eyes, ears or other tissues, or when fixed brain or decalcified head or spine is sectioned. Small or infiltrative lesions, (such as hematopoietic neoplasms infiltrating from marrow, or squamous cell carcinoma invading from oral cavity, may be difficult to identify without a microscope.

Histopathology Findings: Neoplasia – more below.

Nature Influences: Mouse strain, sex and specific mutations (or polymorphisms) have been associated with susceptibility (higher incidence, multiplicity or accelerated onset) or resistance (lower incidence, multiplicity or later onset) of specific types of neoplasms (see below). Primary brain tumors are unusual in unmanipulated mice (Morgan et al., 1984). Hematopoietic neoplasms (commonly lymphoma, histiocytic sarcoma) in marrow can invade adjacent tissues including muscle, spine, brain (Ceccarelli & Rozengurt, 2002). Squamous cell carcinoma of oral cavity or face can be highly locally invasive. Osteosarcomas and poorly differentiated sarcomas as in Tp53 mutant mice can involve spine (Mitchel et al., 2003).

Nurture Influences: Age and exposure to carcinogens. Subcutaneous transponders have been associated with sarcomas in Trp53 mutant mice (Blanchard et al., 1999). Ear tags have been associated with auricular squamous cell carcinoma in FVB/N mice (Baron et al., 2005).

3.18 Condition: Skin, Dermatitis (Mouse Ulcerative Dermatitis, MUD)

Clinical/Behavioral Phenotype(s): Intense scratching, self-mutilation, may be constant or nearly so in the cage and under test conditions.

Gross Pathology Findings (dysmorphology): Ulcerated skin lesions, some appear wet or scalded (like a burn). Groups of mice may have primary involvement of different anatomic sites such as ears and head, dorsum, or ventral neck. Secondary phenotypes include lymphadenomegaly, splenomegaly.

Clinical Pathology: Secondary reactive changes such as neutrophilic and or lymphocytic leukocytosis should be expected.

Histopathology Findings: Ulcers and dermatitis of varying degrees and chronicity, with varying bacterial infiltration and colonization; fibrosis (scarring) and adjacent hyperplastic epithelial changes in chronic conditions. Secondary reactive changes are likely, such as lymphoid hyperplasia in lymph nodes and spleen, myeloid hyperplasia in spleen and bone marrow (Gruys, Tooten, & Kuijpers, 1996; Maita et al., 1988).

Nature Influences: Various. Commonly reported in C57BL with female predisposition (Kastenmayer, Fain, & Perdue, 2006), but seen in almost any background (Slattum et al., 1998). Genetic susceptibilities to hypersensitivity and atopic dermatitis are suspected.

Nurture Influences: Mites should be ruled out as a cause or contributors (Jungmann et al., 1996; 1996). Conspecific aggression and trauma should be ruled out as contributors (Litvin et al., 2007; Van Loo et al., 2001). Diet and allergens have been suspected. Bacteria may contribute to lesion severity, but usually are considered to be opportunistic invaders.

3.19 Condition: Barbering

Clinical/Behavioral Phenotype(s): Barbering seems to be a complex social behavior. Removal of vibrissae (by barbering) may impact tests where discrimination of object, textures, balance, orientation and exploration are mediated or facilitated by vibrissae. Vibrissae are sensory hairs that originate in sinus follicles (Kalueff et al., 2006; Sarna, Dyck, & Whishaw, 2000).

Gross Pathology Findings (dysmorphology): Missing vibrissae; hair loss (alopecia) patterns may be sex and strain specific.

Histopathology Findings: Usually not impressive. Blunt ended hairs indicate nibbling. Possible trauma from plucked hairs or vibrissae may indicate a dominance behavior. Inflammatory changes may suggest alopecia areata or other causes of alopecia (McElwee et al., 2003; 2003; Sun et al., 2008).

Nature Influences: Females are commonly implicated as the primary social barbers. Strain related patterns are reported. Plucking and nibbling (or trimming) are reported (Garner et al., 2004; Kalueff et al., 2006; Sarna et al.,

2000). CH/HeJ mice can develop immune mediated destruction of hair follicles (not associated with barbering) that shares features with human alopecia areata (McElwee et al., 2003; 2003; Sun et al., 2008).

Nurture Influences: Social hierarchies, enrichments, environmental stressors may influence barbering. Mites should be ruled out as a cause or contributor to pruritus, hair loss, dermatitis.

3.20 Mouse Tumor Phenotypes

Common spontaneous neoplasms in mice are hematopoietic, lung, liver, mammary, pituitary tumors. Incidence and onset depend on the mouse strains and conditions of the study (Haines, Chattopadhyay, & Ward, 2001; Haseman, Hailey, & Morris, 1998; Mahler et al., 1996; Maronpot, Boorman, & Gaul, 1999; NTP, 2003; Ward, 2006; Ward et al., 2000). Dietary caloric restriction generally extends lifespan, and delays and/or reduces tumor onset (Blackwell et al., 1995; Bronson & Lipman, 1991; Dirx et al., 2003; Hursting et al., 2004; Lipman, 2002; Mai et al., 2003). Housing density and parity also affect tumors. Various other environmental factors may influence tumor incidence and onset, and should be considered in experimental design, including whether or how to use historical control data (Boorman et al., 2002; Haseman, Boorman, & Huff, 1997; Haseman et al., 1989; 1997; Keenan et al., 2009; Lipman et al., 1993; Martin et al., 2010). In contemporary mouse colonies it may be of interest or concern that infectious agents like helicobacters could contribute to cancer incidence (Diwan et al., 1997; Engle et al., 2002; Hale et al., 2007), and that agents like Parvoviruses, which target cells in S phase of the cell cycle, have been investigated as therapeutic oncolytic agents (Dupressoir et al., 1989; Lang et al., 2006; Malerba et al., 2003).

Experimental designs vary widely among studies and should be evaluated critically when comparing results. Some chronic or aging studies are terminated at 18 months or 2 years. Others evaluate mice at (after) spontaneous death. Dissection, processing and evaluation techniques vary, resulting in examination of different areas, and different absolute and relative amounts of tissues. Small tissues like pituitary, thyroid, adrenals are challenging to dissect and evaluate in every animal. Larger tissues like liver and lung are challenging to evaluate thoroughly and consistently among all animals in a study.

Interpretation and terminology vary among evaluators and has led to efforts for consensus in diagnostic criteria and terminology including the Mouse Models of Human Cancer Consortium (MMHCC) http://emice.nci.nih.gov/ and consensus papers, the European mutant mouse pathology database (Pathbase) http://eulep.pdn.cam.ac.uk/Links/index.php (Sundberg, Sundberg, & Schofield, 2008; Sundberg et al., 2009), and International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) project (http://www.toxpath.org/nomen/), promulgated by the toxicologic pathology community, including the Japanese Society of Toxicologic Pathology (JSTP), the British Society of Toxicologic Pathology (BSTP), the European Society of Toxicologic

Pathology (ESTP), and the Society of Toxicologic Pathology (STP) (Bach et al., 2010; Vahle et al., 2009), and linked to RENI (reference for nomenclature and diagnostic criteria in toxicologic pathology http://www.goreni.org/).

3.21 Condition: Hematopoietic Neoplasia

Clinical Phenotype: Subclinical to weak or moribund mice, wasting, pallor (pale ears, paws, eyes, associated with marrow involvement and replacement of hematopoietic tissue); dyspnea (associated with thymus enlargement).

Gross Pathology Findings (dysmorphology): Gross findings usually include one or more of the following: lymphadenomegaly (pale soft tumor masses around neck, axilla, groin, knee, abdomen); splenomegaly sometimes with prominent pale nodules; hepatomegaly; enlarged pale thymus; ascites. Watery blood may correlate with anemia, thrombocytopenia, and atypical circulating cells may be identified in blood films.

Histopathology Findings: Neoplastic monomorphic-pleomorphic mononuclear cells proliferating in and effacing architecture in almost any tissue especially thymus, lymph nodes, spleen, liver, likely to indiscriminately invade adjacent tissues. Bone marrow and blood tend to be involved late (in advanced disease) by lymphoma or histiocytic sarcoma. Most myeloid neoplasms or leukemias (other than histiocytic sarcoma) are expected to manifest with blood and marrow involvement (Kogan et al., 2002; Morse et al., 2002; Rowe & Pincus, 1972).

Nature Influences: AKR (Stoye, Moroni, & Coffin, 1991; Ward, 2006), C58 (Mucenski et al., 1988), NODscid (Chiu et al., 2002; Serreze et al., 1995) have high incidence and early onset of thymic T lymphoblastic lymphoma. SJL/J have high incidence of a later onset B cell lymphoma, progressing from multicentric polyclonal B cell proliferation (Crispens, 1973; Tang et al., 1998; 1998). Some CFW lines have a high incidence of a spinal lymphoma (Ceccarelli & Rozengurt, 2002). Expression and interactions of endogenous murine leukemia viruses (MuLV, Emv, Xmv) and Mtv's have been shown to cause some of these lymphomas, via insertional activating mutations in cellular proto-oncogenes, or disruptions in tumor suppressor genes or pathways (Hartley et al., 2000; Stoye & Coffin, 1988; Stoye et al., 1991; Taddesse-Heath et al., 2000; Weiser et al., 2007) Ras activation is a major mechanism in development of thymic lymphomas induced or accelerated by radiation and chemical carcinogens (Shimada et al., 2003). Plasmacytoma is more common and more easily induced in BALB/c than in other common strains, and has led to their utility in monoclonal antibody production (Hendriksen & de Leeuw, 1998; Kobayashi, Potter, & Dunn, 1962; Potter & Maccardle, 1964; Potter, Wax, & Blankenhorn, 1985). Lymphoma and histiocytic sarcoma are among the most common tumors in chronic (long term) toxicology studies usually involving Swiss stocks or B6C3F1 mice, as well as in chronic or aging studies of inbred strains including C57BL/6, 129; FVB (Haines

et al., 2001; Haseman et al., 1998; Maronpot et al., 1999; Son & Gopinath, 2004; Ward, 2006). Non lymphoid hematopoietic neoplasia (other than histiocytic sarcoma) are unusual spontaneous tumors in common inbred strains (Kogan et al., 2002; Ward, 2006). Erythroleukemia and myelodysplasia are expected findings in Tg.AC transgenic mice with constitutively activated Ras protooncogene (Bach et al., 2010).

Nurture Influences: Chemical carcinogens and irradiation can induce lymphoma, especially thymic lymphoma (Utsuyama & Hirokawa, 2003). Exposure to horizontally transmitted murine retroviruses via inoculated cell lines can cause lymphoma (Hartley et al., 2008). Murine herpesviruses from wild mice may cause or contribute to an Epstein Barr virus -like disease or leukemia in experimentally infected mice (Hausler et al., 2005; Pappova et al., 2004). Other infections may contribute to incidence of lymphomas (Baird et al., 1982). Specific Pathogen Free (SPF) BALB/c mice may be less likely to develop plasmacytoma than conventionally maintained BALB/c mice (Byrd et al., 1991). In rats, "lymphoma" involving the lung was diagnosed commonly in colonies with respiratory mycoplasmosis. *Mycoplasma pulmonis* and CARbacillus are strong lymphocyte mitogens. Pulmonary lymphoproliferative conditions should be interpreted with caution in colonies known to harbor *M. pulmonis* or CARbacillus (Schoeb et al., 2009).

3.22 Condition: Lung Tumors

Clinical Phenotype: Subclinical to dyspnea, loss of body condition.

Gross Pathology Findings (dysmorphology): Lung nodules, usually pale, soft, single or multiple.

Histopathology Findings: Histology is required to distinguish primary lung neoplasms from metastases, and to distinguish types of primary lung tumors. In contrast to humans, hyperplasia and adenoma are the more common proliferative lesions of the mouse lung. In hyperplastic lesions, alveoli are lined by a uniform population of hypertrophic (plump, bulging) cuboidal cells. The common spontaneous (and chemically induced) lung tumors in mice originate peripherally in lung (bronchoalveolar regions), but the cell of origin is generally unclear (e.g. type II pneumocytes, Clara cells, pluripotent stem cells). NTP and INHAND international harmonization classifications call these A/B (Dixon et al., 2008) and bronchiolo-alveolar adenomas and carcinomas (Bube et al., 2010; Renne et al., 2009), but MMHC withholds the term bronchioloalveolar to avoid confusion with human bronchioloalveolar carcinoma (BAC) (Galvez et al., 2004; Nikitin et al., 2004). Lung, adenocarcinoma, mixed (referring to the growth pattern), would be an example of a MMHCC-type diagnosis. Further characterization could be added to the diagnosis after confirmation of the cell of origin, e.g. Lung, adenocarcinoma, mixed, type II pneumocyte.

Adenomas are discrete, non invasive, compressive, often less than 5 mm diam. Neoplastic cells are generally monomorphic, cuboidal cells on fine stroma tending to retain alveolar features, but can fill alveolar spaces, effacing normal architecture. Mitotic activity is usually low, and there is little atypia. Growth patterns may be papillary, solid or mixed. Carcinomas are usually more than 5 mm diam, exhibit invasion of airways, blood or lymphatic vessels, regional and distant metastasis. They usually have more nuclear and cellular pleomorphism or atypia, more mitotic activity, multiple or variable growth patterns, or necrosis (Dixon et al., 2008; Hahn & Boorman, 1997; Nikitin et al., 2004).

Nature Influences: Lung tumors are expected in A strain mice, with up to 100% incidence by 18–24 months, and also are very common in SWR mice. They are less frequent in BALB/c, 129, FVB/N, CD-1 and other strains or stocks but still may be among their most common neoplasms. C57BL/6 and DBA strains have been found to be quite resistant to development of lung tumors (Abell et al., 1965; Mahler et al., 1996; Malkinson & Thaete, 1986; Rosenzweig & Blaustein, 1970). At least 12 "pulmonary adenoma susceptibility" (Pas) loci and 30 "susceptibility to lung cancer" (Sluc) loci have been identified in mice (Thaete, Nesbitt, & Malkinson, 1991). Different K-ras alleles (polymorphisms) have been associated with differences in mouse lung tumor susceptibility (Chen et al., 1994). Mutations that activate Ras and other oncogenes, or inactivate Tp53 or other tumor suppressor genes can be common, especially in chemically induced lung tumors in mice (Galvez et al., 2004; Meuwissen & Berns, 2005).

Nurture Influences: Chemical carcinogens, including inhaled components of cigarette smoke, welding fumes, etc induce lung tumors in mice. Activating mutations of cellular oncogenes, especially Kras (K-ras), or inactivating mutations of tumor suppressor genes, especially Tp53, can be identified in many induced tumors, with molecular and biological relevance to human pulmonary adenocarcinoma (Dixon et al., 2008; Wakamatsu et al., 2007). A variety of environmental factors should be considered as potential influences on the development of neoplasia in rodents (Keenan et al., 2009). Chronic or healing phases of Sendai virus infections, can include intensely proliferative epithelial lesions. Sendai virus is a Paramyxovirus similar to Parainfluenza 1 virus. It is uncommon in contemporary colonies (Brownstein, Smith & Johnson, 1981; Pritchett-Corning, Cosentino, & Clifford, 2009; Schoondermark-van de Ven, E.M., I.M. Philipse-Bergmann, & J.T. van der Logt, 2006).

3.23 Condition: Mammary Tumors

Clinical Phenotype: Small to large, single to multiple masses almost anywhere on body. Very large tumors may ulcerate.

Gross Pathology Findings (dysmorphology): Subcutaneous nodule (s), single or multiple, usually pale, soft, may be cystic, originating in mammary tissue, which extends from the ears to the tail. Salivary gland tumors, possibly lymphadenomegaly due to lymphoma, or abscesses are differential diagnostic considerations for masses around the neck. The preneoplastic lesions, hyperplastic alveolar nodules or HAN (nodules usually less than 2 mm diameter), or plaques (small flat plaque like lesions in pregnant mice) may be palpable (Cardiff et al., 2007).

Histopathology Findings: Histology, and sometimes immunohistochemistry, is required to distinguish mammary from salivary gland tumors and other tumors in skin and subcutis. Hyperplastic alveolar nodules (HAN), and plaques, formerly called type P tumors, are considered to be preneoplastic. These are transplantable clonal lesions that can progress to invasive, hormone independent malignancy. HAN are foci of alveolar hyperplasia, or clusters of budding acini, with high mitotic rate, usually less than 2 mm diameter. Plaques are circumscribed flat (plaque-like) ductal proliferations with abundant stroma, which occur during pregnancy, and typically regress after parturition. Adenomas are discrete and non invasive, well differentiated tumors that do not progress to malignancy. Large focal tumors with no evidence of metastases or invasion are considered adenomas. Invasion, metastasis, and transplantability are criteria of malignancy (Cardiff et al., 2000; 2007).

Common histologic patterns in spontaneous (MMTV-induced) tumors were called A, B (and P for plaque). The "Annapolis" report of the MMHCC classification advocates morphologic descriptors that correlate with these, but also with morphology of novel genetically engineered neoplasms, adds descriptors for distribution, biological potential (adenoma, adenocarcinoma), and molecular etiology, and adds MIN. Mammary intraepithelial neoplasia (MIN) in GEM are intraepithelial, pre invasive, morphological intermediates between normal tissue and cancer, with characteristics of benign and malignant cells including nuclear atypia. Morphologic (pattern) descriptors in the Annapolis report include acinar, glandular, cribriform (sieve-like), papillary, solid, squamous, adenosquamous, also adenomyoepithelioma and carcinosarcoma as diagnostic entities. The acinar (formerly type A) pattern consists of small similar clusters of similar cells organized around central lumina (resembling small acini or glands). Their nuclei are generally small and oval. An example of Annapolis nomenclature for a spontaneous (MMTV induced) malignant neoplasm of this type would be: Acinar carcinoma, low grade, MMTV induced. Glandular patterns consist primarily of larger glandular structures. Type B were the mixed tumors lacking a dominant pattern, but often a mixture of solid, papillary, acinar. Type C tumors had cystic features, and usually identified in older virus-free mice. Adenosquamous neoplasms (formerly adenoacanthomas) consist of intimately mixed glandular and squamous (often cornified) epithelium. Poorly differentiated sarcomatous tumors previously classified as carcinosarcomas

currently are considered to be epithelial to mesenchymal transition (EMT) tumors, expected to be immunoreactive for intermediate filaments, cytokeratin, and vimentin and loss of E-cadherin (Bittner, 1942; 1956; Dunn, 1958; Kilham, 1952).

Mammary hyperplasia related to nursing or systemic influences are usually generalized, but hormone or pregnancy induced plaques (formerly type P tumors) consist of focally proliferative ducts and connective tissue.

Metastases are not very common, usually to lung, often as intravascular tumor emboli (Cardiff et al., 2000).

Nature Influences: Spontaneous mammary tumors occur almost exclusively in female mice, and are especially common in C3H mice, which were developed to study mammary tumors, and led to the discovery of the mouse mammary tumor virus (MMTV), or Bittner agent (Bittner, 1942; 1956; Dunn, 1958; Kilham, 1952). The exogenous MMTV, transmitted vertically via milk, has been eliminated intentionally from most contemporary mouse strains by fostering or rederivation, but most strains carry endogenous proviruses (Mtv1–55) with variable expression and influences on tumorigenesis (Morse, 2005). MMTV integrations can activate multiple genes including, Wnt, Fgf3, Notch (insertional activation of oncogenes). The CBA strain was developed in conjunction with the C3H strain by selecting for and breeding animals that did not develop mammary tumors, and continue to be long lived mice, with generally low incidence of neoplasia of any kind. BALB/c mice can have relatively high incidence of mammary tumors and salivary tumors. FVB/N females can develop multifocal – widespread mammary hyperplasia, which may interfere with breast cancer modeling on this background. FVB/N mammary tumors have been associated with pituitary prolactinomas, and may feature epithelial-mesenchymal transition (EMT), and estrogen receptor alpha (ERalpha)-positivity (Radaelli et al., 2009),contrasting with many of the spontaneous (MMTV induced) mouse mammary tumors, which are ER-alpha negative and hormone independent (Cardiff et al., 2000; Damonte et al., 2007; Radaelli, Damonte, & Cardiff, 2009; Radaelli et al., 2009).

Nurture Influences: Various carcinogens increase incidence of mammary tumors, e.g. by mutational activation of wnt, ras and other pathways, or inactivation of tumor suppressors like p53 (Hoenerhoff et al., 2009). Some carcinogens induce more adenosquamous or undifferentiated tumors, in contrast to the acinar or mixed patterns associated with MMTV and disruptions in wnt pathway. Various other environmental factors may influence tumor incidence and onset, and should be considered in experimental design (Haseman et al., 1997; 1989; 1997; Keenan et al., 2009). FVB/N mammary changes have been shown to be influenced by substrain, age, environment, diet, parity, pups suckling activity, murine mammary tumor virus (MMTV) infection, and concurrent diseases (Radaelli et al., 2009).

3.24 Condition: Liver Tumors

Clinical Phenotypes: Loss of body condition, possible abdominal mass.

Gross Pathology Findings (dysmorphology): Liver masses, discrete and nodular, to large and coalescing, hepatomegaly in advanced disease.

Histopathology Findings: Histopathology Findings: INHAND classifications of hepatocyte alteration and proliferative lesions in mice similar to those for rat are being developed for mice. Foci of cellular alteration are less common in mice than in rats, more common in male than female, eosinophilic and clear cell foci may exceed basophilic and mixed foci. Usually their size is less than several lobules, and cells merge with adjacent plates/cords without compression of adjacent parenchyma. Cells in clear cell foci have increased cytoplasmic glycogen. Focal fatty change is distinguished by more discrete lipid vacuoles. Regenerative hyperplasia is characterized by discrete nodules of proliferating hepatocytes, and evidence of prior damage. Hepatocellular adenoma is the most common neoplasm in C3H and B6C3F1 mice. They are usually solitary but may be multiple. They are well circumscribed, compressive, and greater than one lobule in size, usually lack central veins and portal tracts unless entrapped. Their hepatocytes are well differentiated but can vary in size and staining, and have low-variable atypia and mitotic activity. They may appear solid but usually are arranged in plates 1–3 layers thick, with compressed sinusoids. Especially in older mice adenomas can be larger, have atypia, degenerative changes, or carcinomas arising in them. Hepatocellular carcinomas usually are spherical and partially well demarcated, with areas of irregular borders due to local invasion and compression. Cellular atypia and mitoses can be prominent. Nuclei usually are large and hyperchromatic with prominent central nucleoli. Growth patterns vary including trabecular, glandular, solid. Acinar or glandular patterns are less common. Metastasis is not very common but usually to lung (Bach et al., 2010; Brix et al., 2005; Maronpot et al., 1987).

Nature Influences: Spontaneous liver tumors are much more common in male than in female mice, more common in C3H and CBA than other common mouse strains (Tillmann, Kamino, & Mohr, 2000). Hepatocarcinogen sensitivity (*Hcs*) loci and other genes are involved in liver carcinogenesis and sensitivity to carcinogens (Feo et al., 2006). Activating ras mutations are commonly identified in spontaneous and carcinogen induced mouse liver tumors but their frequency and roles are influenced by the mouse strain and carcinogen exposure (Maronpot et al., 1995).

Nurture Influences: Carcinogens generally increase and accelerate liver tumor development in susceptible mouse strains. Carcinogen induced mouse liver tumors induced may have increased frequency of mutations in *Hras*, or mutations in beta catenin *Catnb*, depending on the carcinogen (Hoenerhoff et al., 2009). Caloric restriction generally reduces tumor incidence, separate

housing increases tumor incidence, and other environmental factors may influence tumor incidence (Haseman et al., 1997; 1989; 1997; Keenan et al., 2009). Helicobacter hepaticus was "discovered" in a study in which A/J mice (expected to be resistant to development of liver tumors) developed unexpectedly high incidence of liver tumors (Ward et al., 1994).

3.25 Condition: Tumors that are Not Neoplastic (abscesses and inflammation)

Clinical Phenotype: Masses, skin, subcutis various tissues, possible leukocytosis lymphadenomegaly.

Gross Pathology Findings (dysmorphology): Masses, skin, subcutis and other sites that exude pus when expressed or sectioned; possible lymphadenomegaly, splenomegaly.

Histopathology Findings: Abscesses, neutrophilic exudate, bacterial colonies; colonies of bacteria surrounded by eosinophilic (Splendore Hoeppli) material have been called botryomycosis; gram positive cocci consistent with staphylococci are common in these lesions. Lymphadenomegaly, splenomegaly frequently is reactive lymphoid hyperplasia, with prominent myeloid granulocytic hyperplasia in spleen and bone marrow (Bingel, 2002; Bradfield et al., 1993).

Nature Influences: Genetically determined or influenced immune deficiency or deviant immune responses may contribute to morbidity or mortality from opportunistic agents.

Nurture Influences: S aureus has been a common cause of abscesses, but coagulase negative staphylococci and other agents are also implicated especially in immune deficient mice (Akiyama et al., 1996; Bingel, 2002; Bradfield et al., 1993; Clarke et al., 1978; Elliott, Brook and Stiefel, 1990; Girgis et al., 2004; Hong and Ediger, 1978; Lambe et al., 1990; Nagase et al., 2002; Rich and Lee, 2005; Sharmin et al., 2004; Thornton et al., 2003; Won et al., 2002). Streptobacillus moniliformis is an unlikely cause in contemporary colonies and is a zoonotic concern (Glastonbury, Morton, & Matthews, 1996).

In conclusion Genetically engineered mice, like other laboratory animals, clinical patients (human and veterinary), and the rest of us benefit and suffer from the influences of genetic backgrounds, and environmental factors. Pathology can confirm and characterize expected phenotypes, and diagnose unexpected phenotypes. Scientists and science will benefit from better understanding of nature and nurture influences, and from strategic use of pathology to confirm important phenotypes and to investigate unexpected phenotypes. Experimental design for preclinical translational research should include consideration of the genetic backgrounds, diets, bioburden, and other environmental impacts on the selected model, and early implementation of pathology to optimize specimens for analysis, and the contributions of pathology to research success.

3.26 Final Considerations

1. What Mice do you use? Why were these selected?
2. Where do they come from? Why did you select this source?
3. What are complete names of experimental and control mice, including substrain and origin?
4. Do your experimental and control mice live together? In the same cages (e.g. littermates)? In the same rooms? In the same facilities?
5. What is their microbial status? and surveillance program?
6. What do they eat? Brand and type? Percent and type of protein? Percent and type of fat? How is it monitored for contaminants?
7. What do they sleep on (what type of bedding)? Paper? Wood?
8. Do you provide enrichment material or devices? Why or Why not?
9. What do they drink? Auto? Bottle? pH? Chlorine levels? How is it monitored for contaminants?
10. What is their light cycle? Why?
11. What are environmental noise levels and ranges? Does it matter to your study?

3.27 General Mouse Pathology & Phenotyping References

Barthold, S. W. (2004). Genetically altered mice: phenotypes, no phenotypes, and Faux phenotypes. *Genetica*, *122*(1), 75–88.

Brayton, C., Justice, M., Montgomery, C. (2001). Evaluating mutant mice: anatomic pathology. *Veterinary Pathology*, *38*(1), 1–19.

Brayton, C. (2006). Spontaneous diseases in commonly used inbred mouse strains. In J. G. Fox et al. (Eds.), *The mouse in biomedical research* (2nd ed.). ACLAM Series. Amsterdam: Elsevier.

Elmore, S. A. (2006). Histopathology of the lymph nodes. *Toxicologic Pathology*, *34*(5), 425–454. Accessed March 1, 2010, from http://tpx.sagepub.com/cgi/content/full/34/5/425

Elmore, S. A., & Peddada, S. D. (2009). Peddada, points to consider on the statistical analysis of rodent cancer bioassay data when incorporating historical control data. *Toxicologic Pathology*, *37*(5), 672–676.

Frith, C. H., & Ward, J. M. (1988). *A color atlas of neoplastic and non neoplastic lesions in aging mice.* Amsterdam/New York: Elsevier. (Print on demand available through the Charles Louis Davis Foundation at http://www.cldavis.org/). Electronic version available online at www.informatics.jax.org/frithbook/chapters/references.shtml

Hrabé de Angelis, M., Chambon, P., & Brown, S. (Eds.) (2006). *Standards of mouse model phenotyping*. Weinheim: Wiley-VCH, ISBN 3-527-31031-2.

Keenan, C., Elmore, S., Francke-Carroll, S., Kemp, R., Kerlin, R., & Peddada (2009). Best practices for use of historical control data of proliferative rodent lesions. *Toxicologic Pathology*, *37*(5) 679–693.

Kuper, C. F. (2006). Histopathology of mucosa-associated lymphoid tissue. *Toxicologic Pathology*, *34*(5), 609–615. Accessed March 1, 2010, from http://tpx.sagepub.com/cgi/content/full/34/5/609

Maronpot, R. R., Boorman, G. A., & Gaul, B. W. (Eds.). (1999). *Pathology of the mouse: Reference and atlas*. Vienna, IL: Cache River Press.

Papaioannou, V. E., & Behringer R. R. (2004). *Mouse phenotypes: A Handbook of mutation analysis*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Pearse, G. (2006). Histopathology of the thymus. *Toxicologic Pathology*, *34*(5), 515–547. Accessed March 1, 2010, from http://tpx.sagepub.com/cgi/content/full/34/5/515

Suttie, A. W. (2006). Histopathology of the spleen. *Toxicologic Pathology*, *34*(5), 466–503. Accessed March 1, 2010, from http://tpx.sagepub.com/cgi/content/full/34/5/466

Travlos, G. S. (2006). Histopathology of bone marrow. *Toxicologic Pathology*, *34*(5), 566–598. Accessed March 1, 2010, from http://tpx.sagepub.com/cgi/content/full/34/5/566

Ward, J. M., Anver, M. R., Mahler, J. F., & Devor-Henneman, D. E. (2000). Pathology of mice commonly used in genetic engineering (C57BL/6; 129; B6;129; FVB). In M. J. Ward, J. M., Maronpot, R. R., Sundberg, J. P. (Eds.), *Pathology of genetically engineered mice*. Ames, IA: Iowa State University Press.

References

Abell, C. W., et al. (1965). Uracil mustard: A potent inducer of lung tumors in mice. *Science*, *147*, 1443–1445.

Akiyama, H., et al. (1996). Staphylococcus aureus infection on cut wounds in the mouse skin: Experimental staphylococcal botryomycosis. *The Journal of Dermatological Science*, *11*(3), 234–238.

Anderson, M. G., et al. (2008). GpnmbR150X allele must be present in bone marrow derived cells to mediate DBA/2 J glaucoma. *BMC Genetics*, *9*, 30.

Azoulay-Cayla, A., et al. (2001). Roles of the H-2Db and H-Kb genes in resistance to persistent Theiler's murine encephalomyelitis virus infection of the central nervous system. *Journal of General Virology*, *82*(5), 1043–1047.

Bach, U., et al. (2010). Proceedings of the 2009 national toxicology program satellite symposium. *Toxicologic Pathology*, *38*(1), 9–36.

Bailey, K. R., Rustay, N. R., & Crawley, J. N. (2006). Behavioral phenotyping of transgenic and knockout mice: Practical concerns and potential pitfalls. *ILAR Journal*, *47*(2), 124–131.

Baird, S. M., et al. (1982). Induction of lymphoma in antigenically stimulated athymic mice. *Cancer Research*, *42*(1), 198–206.

Balkema, G. W., & Drager, U. C. (1991). Impaired visual thresholds in hypopigmented animals. *Visual Neuroscience*, *6*(6), 577–585.

Balogh, S. A., et al. (1999). A behavioral and neuroanatomical assessment of an inbred substrain of 129 mice with behavioral comparisons to C57BL/6 J mice. *Brain Research*, *836*(1–2), 38–48.

Baron, B. W., et al. (2005). Squamous cell carcinomas of the skin at ear tag sites in aged FVB/N mice. *Comparative Medicine*, *55*(3), 231–235.

Barthold, S. W. (2002). "Muromics": Genomics from the perspective of the laboratory mouse. *Comparative Medicine*, *52*(3), 206–223.

Beckmann, N. (2000). High resolution magnetic resonance angiography non-invasively reveals mouse strain differences in the cerebrovascular anatomy in vivo. *Magnetic Resonance in Medicine*, *44*(2), 252–258.

Bingel, S. A. (2002). Pathology of a mouse model of x-linked chronic granulomatous disease. *Contemporary Topics in Laboratory Animal Science*, *41*(5), 33–38.

Bittner, J. J. (1942). The milk-influence of breast tumors in mice. *Science*, *95*(2470), 462–463.

Bittner, J. J. (1956). Mammary cancer in C3H mice of different sublines and their hybrids. *Journal of the National Cancer Institute*, *16*(5), 1263–1286.

Blackwell, B. N., et al. (1995). Longevity, body weight, and neoplasia in ad libitum-fed and diet-restricted C57BL6 mice fed NIH-31 open formula diet. *Toxicologic Pathology*, *23*(5), 570–582.

Blanchard, K. T., et al. (1999). Transponder-induced sarcoma in the heterozygous p53+/− mouse. *Toxicologic Pathology*, *27*(5), 519–527.

Bleich, A., et al. (2008). Klebsiella oxytoca: Opportunistic infections in laboratory rodents. *Laboratory Animal*, *42*(3), 369–575.

Bloom, D., & Hultcrantz, M. (1994). Vestibular morphology in relation to age and circling behavior. *Acta Otolaryngologica*, *114*(4), 387–392.

Boorman, G. A., et al. (2002). Quality review procedures necessary for rodent pathology databases and toxicogenomic studies: The national toxicology program experience. *Toxicologic Pathology*, *30*(1), 88–92.

Bradfield, J. F., et al. (1993). Epizootic fatal dermatitis in athymic nude mice due to *Staphylococcus xylosus*. *Laboratory Animal Scencei*, *43*(1), 111–113.

Brayton, C., Justice, M., & Montgomery, C. A. (2001). Evaluating mutant mice: Anatomic pathology. *Veterinary Pathology*, *38*(1), 1–19.

Brix, A., et al. (2005). *A digitized atlas of rat liver lesions* (Vol. 1, 1st ed.). Research Triangle Park, NC: Laboratory of Experimental Pathology, National Toxicology Program. (Distributed by NIEHS, NTP. Free of Charge)

Brodkin, E. S. (2007). BALB/c mice: Low sociability and other phenotypes that may be relevant to autism. *Behavioural Brain Research*, *176*(1), 53–65.

Bronson, R. T., & Lipman, R. D. (1991). Reduction in rate of occurrence of age related lesions in dietary restricted laboratory mice. *Growth Development & Aging*, *55*(3), 169–184.

Brown, R. E., & Wong, A. A. (2007). The influence of visual ability on learning and memory performance in 13 strains of mice. *Learning & Memory*, *14*(3), 134–144.

Brownstein, D. G., Smith, A. L., & Johnson, E. A. (1981). Sendai virus infection in genetically resistant and susceptible mice. *The American Journal of Pathology*, *105*(2), 156–163.

Bube, A., et al. International Harmonization of Rat Nomenclature. Joint STPs/ILSI Committee on International Harmonization of Nomenclature and Diagnostic Criteria in Toxicologic Pathology 2000 [cited 2010 March 12; Classification system]. Available from http://www.toxpath.org/nomen/

Byrd, L. G., et al. (1991). Specific pathogen-free BALB/cAn mice are refractory to plasmacytoma induction by pristane. *The Journal of Immunology*, *147*(10), 3632–3637.

Calderone, L., Grimes, P., & Shalev, M. (1986). Acute reversible cataract induced by xylazine and by ketamine-xylazine anesthesia in rats and mice. *Experimental Eye Research*, *42*(4), 331–337.

Cardiff, R. D., et al. (2000). The mammary pathology of genetically engineered mice: The consensus report and recommendations from the Annapolis meeting. *Oncogene*, *19*(8), 968–988.

Cardiff, R. D., et al. (2004). Validation: The new challenge for pathology. *Toxicologic Pathology*, *32*(Suppl 1), 31–39.

Cardiff, R. D., et al. (2007). Mouse mammary tumor biology: A short history. In *Advances in cancer research*. Academic Press, *98*, 53–116.

Cardiff, R. D. (2009). How to phenotype a mouse. *Disease Models & Mechanisms*, *2*(7–8), 317–321.

Carton, C. A., et al. (1956) Studies of hydrocephalus in C57 black mice. *Transactions of the American Neurological Association*, (81st Meeting): 147–149.

Ceccarelli, A. V., & Rozengurt, N. (2002). Outbreak of hind limb paralysis in young CFW Swiss Webster mice. *Comparative Medicine*, *52*(2), 171–175.

Chang, B., et al. (1999). Interacting loci cause severe iris atrophy and glaucoma in DBA/2 J mice. *Nature Genetics*, *21*(4), 405–409.

Chang, B., et al. (2002). Retinal degeneration mutants in the mouse. *Vision Research*, *42*(4), 517–525.

Chen, B., et al. (1994). The second intron of the K-ras gene contains regulatory elements associated with mouse lung tumor susceptibility. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(4), 1589–1593.

Chiu, P. P. L., et al. (2002). Susceptibility to lymphoid neoplasia in immunodeficient strains of nonobese diabetic mice. *Cancer Research*, *62*(20), 5828–5834.

Clapcote, S. J., et al. (2005). NIH Swiss and Black Swiss mice have retinal degeneration and performance deficits in cognitive tests. *Comparative Medicine*, *55*(4), 310–316.

Clarke, M. C., et al. (1978). The occurrence in mice of facial and mandibular abscesses associated with *Staphylococcus aureus*. *Laboratory Animal*, *12*(3), 121–123.
Coley, S. E., et al. (2005). Recombinant mouse hepatitis virus strain A59 from cloned, full-length cDNA replicates to high titers in vitro and is fully pathogenic in vivo. *Journal of Virology*, *79*(5), 3097–3106.
Crispens, C. G. (1973). Some characteristics of strain SJL-JDg mice. *Laboratory Animal Science*, *23*(3), 408–413.
Damonte, P., et al. (2007). EMT tumorigenesis in the mouse mammary gland. *Laboratory Investigation*, *87*(12), 1218–1226.
Dandekar, A. A., & Perlman, S. (2002). Virus-induced demyelination in nude mice is mediated by gamma delta T cells. *The American Journal of Pathology*, *161*(4), 1255–1263.
Davis, L. E. (1990). Comparative experimental viral labyrinthitis. *American Journal of Otolaryngology*, *11*(6), 382–388.
Davis, R. R., et al. (2001). Genetic basis for susceptibility to noise-induced hearing loss in mice. *Hearing Research*, *155*(1–2), 82.
Deb, C., et al. (2009). Demyelinated axons and motor function are protected by genetic deletion of perforin in a mouse model of multiple sclerosis. *Journal of Neuropathology and Experimental Neurology*, *68*(9), 1037–1048.
Denenberg, V. H., et al. (1992). A behavior profile of the MRL/Mp lpr/lpr mouse and its association with hydrocephalus. *Brain, Behavior, and Immunity*, *6*(1), 40–49.
Dietrich, H. M., Khaschabi, D., & Albini, B. (1996). Isolation of Enterococcus durans and Pseudomonas aeruginosa in a scid mouse colony. *Laboratory Animal*, *30*(2), 102–107.
Dirx, M. J., et al. (2003). Energy restriction and the risk of spontaneous mammary tumors in mice: A meta-analysis. *International Journal of Cancer*, *106*(5), 766–770.
Diwan, B. A., et al. (1997). Promotion by Helicobacter hepaticus-induced hepatitis of hepatic tumors initiated by N-nitrosodimethylamine in male A/JCr mice. *Toxicologic Pathology*, *25*(6), 597–605.
Dixon, D., et al. (2008). Summary of chemically induced pulmonary lesions in the National Toxicology Program (NTP) toxicology and carcinogenesis studies. *Toxicologic Pathology*, *36*(3), 428–439.
Drage, M. G., Holmes, G. L., & Seyfried, T. N. (2002). Hippocampal neurons and glia in epileptic EL mice. *Journal of Neurocytology*, *31*(8–9), 681–692.
Dunn, T. (1958). Morphology of mammary tumors in mice. In F. Homburger (Ed.), *The physiopathology of cancer* (pp. 38–84). New York: Paul B. Hoeber, Inc.
Dupressoir, T., et al. (1989). Inhibition by parvovirus H-1 of the formation of tumors in nude mice and colonies in vitro by transformed human mammary epithelial cells. *Cancer Res*, *49*(12), 3203–3208.
Elliott, T. B., Brook, I., & Stiefel, S. M. (1990). Quantitative study of wound infection in irradiated mice. *International Journal of Radiation Biology*, *58*(2), 341–350.
Engle, S. J., et al. (2002). Elimination of colon cancer in germ-free transforming growth factor beta 1-deficient mice. *Cancer Research*, *62*(22), 6362–6366.
Engstrom, F. L., & Woodbury, D. M. (1988). Seizure susceptibility in DBA and C57 mice: The effects of various convulsants. *Epilepsia*, *29*(4), 389–395.
Feo, F., et al. (2006). Hepatocellular carcinoma as a complex polygenic disease. Interpretive analysis of recent developments on genetic predisposition. *Biochimica et Biophysica Acta (BBA) – Reviews on Cancer*, *1765*(2), 126.
Filgueiras, C. C., & Manhães, A. C. (2004). Effects of callosal agenesis on rotational side preference of BALB/cCF mice in the free swimming test. *Behavioural Brain Research*, *155*(1), 13.
Fraunfelder, F. T., & Burns, R. P. (1970). Acute reversible lens opacity: Caused by drugs, cold, anoxia, asphyxia, stress, death and dehydration. *Experimental Eye Research*, *10*(1), 19.
Fredrickson, D. S., & Lees, R. S. (1965). A system for phenotyping hyperlipoproteinemia. *Circulation*, *31*, 321–327.

Galvez, J. J., et al. (2004). Mouse models of human cancers (Part 2). *Comparative Medicine*, *54*(1), 13–15.

Garner, J. P., et al. (2004). Barbering (fur and whisker trimming) by laboratory mice as a model of human trichotillomania and obsessive-compulsive spectrum disorders. *Comparative Medicine*, *54*(2), 216–224.

Girgenrath, M., et al. (2009). Pathology is alleviated by doxycycline in a laminin-alpha2-null model of congenital muscular dystrophy. *Annals of Neurology*, *65*(1), 47–56.

Girgis, D. O., et al. (2004). Susceptibility of aged mice to Staphylococcus aureus keratitis. *Current Eye Research*, *29*(4–5), 269–275.

Glastonbury, J. R., Morton, J. G., & Matthews, L. M. (1996). Streptobacillus moniliformis infection in Swiss white mice. *Journal of Veterinary Diagnostic Investigation*, *8*(2), 202–209.

Goelz, M. F., et al. (1996). Efficacy of various therapeutic regimens in eliminating Pasteurella pneumotropica from the mouse. *Laboratory Animal Science*, *46*(3), 280–285.

Goelz, M. F., et al. (1998). Neuropathologic findings associated with seizures in FVB mice. *Laboratory Animal Science*, *48*(1), 34–37.

Golden, G. T., et al. (2001). Acute cocaine-induced seizures: Differential sensitivity of six inbred mouse strains. *Neuropsychopharmacology*, *24*(3), 291–299.

Graw, J. (2009). Mouse models of cataract. *Journal of Genetics*, *88*(4), 469–486.

Greenman, D. L., et al. (1982). Influence of cage shelf level on retinal atrophy in mice. *Laboratory Animal Science*, *32*(4), 353–356.

Gruys, E., Tooten, P. C., & Kuijpers, M. H. (1996). Lung, ileum and heart are predilection sites for AApoAII amyloid deposition in CD-1 Swiss mice used for toxicity studies. Pulmonary amyloid indicates AApoAII. *Laboratory Animal*, *30*(1), 28–34.

Hahn, F. F., & Boorman, G. A. (1997). Neoplasia and preneoplasia of the lung. In P. Bannasch & W. Gossner (Ed.), *Pathology of neoplasia and preneoplasia in rodents EULEP color atlas* (pp. 29–42). Stuttgart: Schattauer.

Haines, D. C., Chattopadhyay, S., & Ward, J. M. (2001). Pathology of aging B6;129 mice. *Toxicologic Pathology*, *29*(6), 653–661.

Hale, L. P., et al. (2007). Neonatal co-infection with helicobacter species markedly accelerates the development of inflammation-associated colonic neoplasia in IL-10(–/–) mice. *Helicobacter*, *12*(6), 598–604.

Hartley, J. W., et al. (2000). Accelerated appearance of multiple B cell lymphoma types in NFS/N mice congenic for ecotropic murine leukemia viruses. *Laboratory Investigation*, *80*(2), 159–169.

Hartley, J. W., et al. (2008). Expression of infectious murine leukemia viruses by RAW264.7 cells, a potential complication for studies with a widely used mouse macrophage cell line. *Retrovirology*, *5*, 1.

Haseman, J. K., et al. (1989). Sources of variability in rodent carcinogenicity studies. *Fundamental and Applied Toxicology*, *12*(4), 793–804.

Haseman, J. K., et al. (1997). Body weight-tumor incidence correlations in long-term rodent carcinogenicity studies. *Toxicologic Pathology*, *25*(3), 256–263.

Haseman, J. K., Boorman, G. A., & Huff, J. (1997). Value of historical control data and other issues related to the evaluation of long-term rodent carcinogenicity studies. *Toxicologic Pathology*, *25*(5), 524–527.

Haseman, J. K., Hailey, J. R., & Morris, R. W. (1998). Spontaneous neoplasm incidences in Fischer 344 rats and B6C3F1 mice in two-year carcinogenicity studies: A National Toxicology Program update. *Toxicologic Pathology*, *26*(3), 428–441.

Hausler, M., et al. (2005). Murine gammaherpesvirus-68 infection of mice: A new model for human cerebral Epstein-Barr virus infection. *Annals of Neurology*, *57*(4), 600–603.

Hegde, K. R., & Varma, S. D. (2005). Cataracts in experimentally diabetic mouse: Morphological and apoptotic changes. *Diabetes, Obesity and Metabolism*, *7*(2), 200–204.

Hendriksen, C. F., & de Leeuw, W. (1998). Production of monoclonal antibodies by the ascites method in laboratory animals. *Research in Immunology*, *149*(6), 535–542.

Hewicker, M., & Trautwein, G. (1987). Sequential study of vasculitis in MRL mice. *Laboratory Animal*, *21*(4), 335–341.

Ho, M., et al. (2004). Disruption of muscle membrane and phenotype divergence in two novel mouse models of dysferlin deficiency. *Human Molecular Genetics*, *13*(18), 1999–2010.

Hoenerhoff, M. J., et al. (2009). A review of the molecular mechanisms of chemically induced neoplasia in rat and mouse models in National Toxicology Program bioassays and their relevance to human cancer. *Toxicologic Pathology*, *37*(7), 835–848.

Holtz, A., Borman, G., & Li, C. P. (1966). Hydrocephalus in mice infected with polyoma virus. *Proceedings of the Society for Experimental Biology and Medicine*, *121*(4), 1196–1200.

Hom, A. C., Leppik, I. E., & Rask, C. A. (1993). Effects of estradiol and progesterone on seizure sensitivity in oophorectomized DBA/2 J mice and C57/EL hybrid mice. *Neurology*, *43*(1), 198–204.

Hong, C. C., & Ediger, R. D. (1978). Preputial gland abscess in mice. *Laboratory Animal Science*, *28*(2), 153–156.

Hubert, M. F., Gerin, G., & Durand-Cavagna, G. (1999). Spontaneous ophthalmic lesions in young Swiss mice. *Laboratory Animal Science*, *49*(3), 232–240.

Hultcrantz, M., & Spangberg, M. L. (1997). Pathology of the cochlea following a spontaneous mutation in DBA/2 mice. *Acta Otolaryngologica*, *117*(5), 689–695.

Hursting, S. D., et al. (2004). Diet-gene interactions in p53-deficient mice: Insulin-like growth factor-1 as a mechanistic target. *Journal of Nutrition*, *134*(9), 2482S–2486S.

Jablonski, M. M., et al. (2005). The Tennessee Mouse Genome Consortium: Identification of ocular mutants. *Visual Neuroscience*, *22*(5), 595–604.

Jasmin, G., & Bajusz, E. (1962). Myocardial lesions in strain 129 dystrophic mice. *Nature*, *193*, 181–182.

Jimenez, A. J., et al. (1996). The spatio-temporal pattern of photoreceptor degeneration in the aged rd/rd mouse retina. *Cell and Tissue Research*, *284*(2), 193–202.

Johannsen, W. (1911). The genotype conception of heredity. *The American Naturalist*, *45*(531), 129.

John, S. W., et al. (1998). Essential iris atrophy, pigment dispersion, and glaucoma in DBA/2 J mice. *Investigative Ophthalmology & Visual Science*, *39*(6), 951–962.

Jones, S. M., et al. (2006). A comparison of vestibular and auditory phenotypes in inbred mouse strains. *Brain Research*, *1091*(1), 40–46.

Jones, B. W., & Marc, R. E. (2005). Retinal remodeling during retinal degeneration. *Experimental Eye Research*, *81*(2), 123.

Jungmann, P., et al. (1996). Murine acariasis: I. Pathological and clinical evidence suggesting cutaneous allergy and wasting syndrome in BALB/c mouse. *Research in Immunology*, *147*(1), 27–38.

Jungmann, P., et al. (1996). Murine acariasis. II. Immunological dysfunction and evidence for chronic activation of Th-2 lymphocytes. *Scandinavian Journal of Immunology*, *43*(6), 604–612.

Kalueff, A. V., et al. (2006). Hair barbering in mice: Implications for neurobehavioural research. *Behavioural Processes*, *71*(1), 8.

Kastenmayer, R. J., Fain, M. A., & Perdue, K. A. (2006). A retrospective study of idiopathic ulcerative dermatitis in mice with a C57BL/6 background. *The Journal of the American Association for Laboratory Animal Science*, *45*(6), 8–12.

Keeler, C. E. (1924). The inheritance of a retinal abnormality in white mice. *Proceedings of the National Academy of Sciences of the United States of America*, *10*(7), 329–333.

Keenan, C., et al. (2009). Best practices for use of historical control data of proliferative rodent lesions. *Toxicologic Pathology*, *37*(5), 679–693.

Keithley, E. M., et al. (2004). Age-related hearing loss and the ahl locus in mice. *Hearing Research*, *188*(1–2), 21–28.

Kilham, L. (1952). Isolation in suckling mice of a virus from C3H mice harboring Bittner milk agent. *Science*, *116*(3015), 391–392.

Kobayashi, H., Potter, M., & Dunn, T. B. (1962). Bone lesions produced by transplanted plasma-cell tumors in BALB/c mice. *Journal of the National Cancer Institute*, *28*, 649–677.

Kogan, S. C., et al. (2002). Bethesda proposals for classification of nonlymphoid hematopoietic neoplasms in mice. *Blood*, *100*(1), 238–245.

Kohn, D. F., & MacKenzie, W. F. (1980). Inner ear disease characterized by rolling in C3H mice. *Journal of the American Veterinary Medical Association*, *177*(9), 815–817.

Kristensson, K., et al. (1984). Sendai virus infection in the mouse brain: Virus spread and long-term effects. *Acta Neuropathol (Berlin)*, *63*(2), 89–95.

Kumar, A. S., et al. (2004). Virus persistence in an animal model of multiple sclerosis requires virion attachment to sialic acid coreceptors. *Journal of Virology*, *78*(16), 8860–8867.

Lagace-Simard, J., Descoteaux, J. P., & Lussier, G. (1980). Experimental pneumovirus infections: 1. Hydrocephalus of mice due to infection with pneumonia virus of mice (PVM). *The American Journal of Pathology*, *101*(1), 31–40.

Lambe, D. W., Jr., et al. (1990). Pathogenicity of *Staphylococcus lugdunensis*, *Staphylococcus schleiferi*, and three other coagulase-negative *staphylococci* in a mouse model and possible virulence factors. *Canadian Journal of Microbiology*, *36*(7), 455–463.

Lang, S. I., et al. (2006). Humoral immune responses against minute virus of mice vectors. *The Journal of Gene Medicine*, *8*(9), 1141–1150.

Lavi, E., et al. (1986). The organ tropism of mouse hepatitis virus A59 in mice is dependent on dose and route of inoculation. *Laboratory Animal Science*, *36*(2), 130–135.

Lefaucheur, J. P., Pastoret, C., & Sebille, A. (1995). Phenotype of dystrophinopathy in old mdx mice. *Anatomical Record 242*(1), 70–76.

Lipman, R. D., et al. (1993). Husbandry factors and the prevalence of age-related amyloidosis in mice. *Laboratory Animal Science*, *43*(5), 439–444.

Lipman, R. D. (2002). Effect of calorie restriction on mortality kinetics in inbred strains of mice following 7,12-dimethylbenz[a]anthracene treatment. *Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *57*(4), B153–B157.

Litvin, Y., et al. (2007). A pinch or a lesion: A reconceptualization of biting consequences in mice. *Aggressive Behavior*, *33*(6), 545–551.

Livy, D. J., & Wahlsten, D. (1991). Tests of genetic allelism between four inbred mouse strains with absent corpus callosum. *Journal of Heredity*, *82*(6), 459–464.

MacArthur, C. J., Pillers, D. A., Pang, J., Degagne, J. M., Kempton, J. B., Trune, D. R. (2008). Gram-negative pathogen Klebsiella oxytoca is associated with spontaneous chronic otitis media in Toll-like receptor 4-deficient C3H/HeJ mice. *Acta oto-laryngologica*, *128*(2), 132–138.

Mahler, J. F., et al. (1996). Spontaneous lesions in aging FVB/N mice. *Toxicol Pathol 24*(6), 710–716.

Mai, V., et al. (2003). Calorie restriction and diet composition modulate spontaneous intestinal tumorigenesis in Apc(Min) mice through different mechanisms. *Cancer Research*, *63*(8), 1752–1755.

Maita, K., et al. (1988). Mortality, major cause of moribundity, and spontaneous tumors in CD-1 mice. *Toxicologic Pathology*, *16*(3), 340–349.

Malerba, M., et al. (2003). Replicating parvoviruses that target colon cancer cells. *Journal of Virology*, *77*(12), 6683–6691.

Malkinson, A. M., & Thaete, L. G. (1986). Effects of strain and age on prophylaxis and co-carcinogenesis of urethan-induced mouse lung adenomas by butylated hydroxytoluene. *Cancer Research*, *46*(4 Pt 1), 1694–1697.

Margolis, G., & Kilham, L. (1969). Hydrocephalus in hamsters, ferrets, rats, and mice following inoculations with reovirus type I. II. Pathologic studies. *Laboratory Investigation*, *21*(3), 189–198.

Maronpot, R. R., et al. (1987). Liver lesions in B6C3F1 mice: The National Toxicology Program. Experience and Position. *Archives of Toxicology. Supplement*, *10*, 10–26.

Maronpot, R. R., et al. (1995). Mutations in the ras proto-oncogene: Clues to etiology and molecular pathogenesis of mouse liver tumors. *Toxicology 101*(3), 125–156.

Maronpot, R. R., Boorman, G. A., & Gaul, B. W. (Eds.). (1999). *Pathology of the mouse: Reference and atlas* (1st ed.). Vienna, IL: Cache River Press.

Martin, B., et al. (2010). "Control" laboratory rodents are metabolically morbid: Why it matters. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(14), 6127–6133.

McElwee, K. J., et al. (2003). Alopecia areata susceptibility in rodent models. *Journal of Investigative Dermatology Symposium Proceedings*, *8*(2), 182–187.

McElwee, K. J., et al. (2003). Alopecia areata in C3H/HeJ mice involves leukocyte-mediated root sheath disruption in advance of overt hair loss. *Veterinary Pathology*, *40*(6), 643–650.

McGinn, M. D., Bean-Knudsen, D., & Ermel, R. W. (1992). Incidence of otitis media in CBA/J and CBA/CaJ mice. *Hearing Research*, *59*(1), 1–6.

McLin, J. P., & Steward, O. (2006). Comparison of seizure phenotype and neurodegeneration induced by systemic kainic acid in inbred, outbred, and hybrid mouse strains. *European Journal of Neuroscience*, *24*(8), 2191–2202.

Meuwissen, R., & Berns, A. (2005). Mouse models for human lung cancer. *Genes & Development*, *19*(6), 643–664.

Meyer, L. M., et al. (2005). UVR-B induced cataract development in C57 mice. *Experimental Eye Research*, *81*(4), 389–394.

Mitchel, R. E., et al. (2003). Low doses of radiation increase the latency of spontaneous lymphomas and spinal osteosarcomas in cancer-prone, radiation-sensitive Trp53 heterozygous mice. *Radiation Research*, *159*(3), 320–327.

Mitchell, C. R., et al. (1997). Otitis media incidence and impact on the auditory brain stem response in lipopolysaccharide-nonresponsive C3H/HeJ mice. *Otolaryngology-Head and Neck Surgery*, *117*(5), 459–464.

Mohajeri, M. H., et al. (2004). The impact of genetic background on neurodegeneration and behavior in seizured mice. *Genes, Brain & Behavior*, *3*(4), 228–239.

Morgan, K. T., et al. (1984). A morphologic classification of brain tumors found in several strains of mice. *Journal of the National Cancer Institute*, *72*(1), 151–160.

Morse, H. C., III, et al. (2002). Bethesda proposals for classification of lymphoid neoplasms in mice. *Blood*, *100*(1), 246–258.

Morse, H. C. I. (2005). Retroelements in the mouse. In J. G. Fox (Ed.), *Biology of the laboratory mouse*. Amsterdam: ACLAM, Elsevier.

Mucenski, M. L., et al. (1988). Comparative molecular genetic analysis of lymphomas from six inbred mouse strains. *Journal of Virology*, *62*(3), 839–846.

Mullink, J. W., & Haneveld, G. T. (1979). Polyarteritis in mice due to spontaneous hypertension. *The Journal of Comparative Pathology*, *89*(1), 99–106.

NTP. (2003) *Historical control information for B6C3F1 Mice*, N.T. Program, Editor, National Toxicology Program: Research Triangle Park, NC. p. 1–160.

Nagase, N., et al. (2002). Isolation and species distribution of staphylococci from animal and human skin. *Journal of Veterinary Medical Science*, *64*(3), 245–250.

Nikitin, A. Y., et al. (2004). Classification of proliferative pulmonary lesions of the mouse: Recommendations of the mouse models of human cancers consortium. *Cancer Research 64*(7), 2307–2316.

Nose, M., et al. (2000). Genetic basis of autoimmune disease in MRL/lpr mice: Dissection of the complex pathological manifestations and their susceptibility loci. *Reviews in Immunogenetics*, *2*(1), 154–164.

Pappova, M., et al. (2004). Pathogenetical characterization of isolate MHV-60 of mouse herpesvirus strain 68. *Acta Virologica*, *48*(2), 91–96.

Pendergrass, W., et al. (2005). Accumulation of DNA, nuclear and mitochondrial debris, and ROS at sites of age-related cortical cataract in mice. *Investigative Ophthalmology & Visual Science*, *46*(12), 4661–4670.

Pittler, S. J., et al. (1993). PCR analysis of DNA from 70-year-old sections of rodless retina demonstrates identity with the mouse rd defect. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(20), 9616–9619.

Poirrier, A. L., et al. Ototoxic drugs: Difference in sensitivity between mice and guinea pigs. *Toxicology Letters*, *193*(1), 41–49.

Potter, M., & Maccardle, R. C. (1964). Histology of developing plasma cell neoplasia induced by mineral oil in Balb/C mice. *Journal of the National Cancer Institute*, *33*, 497–515.

Potter, M., Wax, J. S., & Blankenhorn, E. (1985). BALB/c subline differences in susceptibility to plasmacytoma induction. *Current Topics in Microbiology and Immunology*, *122*, 234–241.

Prince, J. E., et al. (2001). The differential roles of LFA-1 and Mac-1 in host defense against systemic infection with Streptococcus pneumoniae. *The Journal of Immunology*, *166*(12), 7362–7369.

Pritchett-Corning, K. R., Cosentino, J., & Clifford, C. B. (2009). Contemporary prevalence of infectious agents in laboratory mice and rats. *Laboratory Animal*, *43*(2), 165–173.

Puk, O., et al. (2006).Variations of eye size parameters among different strains of mice. *Mammalian Genome*, *17*(8), 851–857.

Qu, W. M., et al. (2000). Genetic dissection of vasculitis in MRL/lpr lupus mice: A novel susceptibility locus involving the CD72c allele. *European Journal of Immunology*, *30*(7), 2027–2037.

Rachel, R. A., et al. (2002). Spatiotemporal features of early neuronogenesis differ in wild-type and albino mouse retina. *The Journal of Neuroscience*, *22*(11), 4249–4263.

Radaelli, E., et al. (2009). Mammary tumor phenotypes in wild-type aging female FVB/N mice with pituitary prolactinomas. *Veterinary Pathology*, *46*(4), 736–745.

Radaelli, E., Damonte, P., & Cardiff, R. D. (2009). Epithelial-mesenchymal transition in mouse mammary tumorigenesis. *Future Oncology*, *5*(8), 1113–1127.

Raphael, Y., et al. (2001). Severe vestibular and auditory impairment in three alleles of Ames waltzer (av) mice. *Hearing Research*, *151*(1–2), 237.

Renne, R., et al. (2009). Proliferative and nonproliferative lesions of the rat and mouse respiratory tract. *Toxicologic Pathology*, *37*(7 Suppl), 5S–73S.

Rich, J., & Lee, J. C. (2005). The pathogenesis of *Staphylococcus aureus* infection in the diabetic NOD mouse. *Diabetes*, *54*(10), 2904–2910.

Ridder, W. H., Nusinowitz, S., & Heckenlively, J. R. (2002). Causes of cataract development in anesthetized mice. *Experimental Eye Research*, *75*(3), 365.

Rosenzweig, S., & Blaustein, F. M. (1970). Cleft palate in A/J mice resulting from restraint and deprivation of food and water. *Teratology*, *3*(1), 47–52.

Rowe, W. P., & Pincus, T. (1972). Quantitative studies of naturally occurring murine leukemia virus infection of AKR mice. *The Journal of Experimental Medicine*, *135*(2), 429–436.

Sarna, J. R., Dyck, R. H., & Whishaw, I. Q. (2000). The Dalila effect: C57BL6 mice barber whiskers by plucking. *Behavioural Brain Research*, *108*(1), 39–45.

Schoeb, T. R., et al. (2009). Mycoplasma pulmonis and lymphoma in bioassays in Rats. *Veterinary Pathology*, *46*(5), 952–959.

Schoondermark-van de Ven, E. M., Philipse-Bergmann, I. M., & van der Logt, J. T. (2006). Prevalence of naturally occurring viral infections, Mycoplasma pulmonis and Clostridium piliforme in laboratory rodents in Western Europe screened from 2000 to 2003. *Laboratory Animal*, *40*(2), 137–143.

Serfilippi, L. M., et al. (2004). Assessment of retinal degeneration in outbred albino mice. *Comparative Medicine*, *54*(1), 69–76.

Serreze, D. V., et al. (1995). Emv30null NOD-scid mice. An improved host for adoptive transfer of autoimmune diabetes and growth of human lymphohematopoietic cells. *Diabetes*, *44*(12), 1392–1398.

Sharmin, S., et al. (2004). *Staphylococcus aureus* antigens induce IgA-type glomerulonephritis in Balb/c mice. *The Journal of Nephrology*, *17*(4), 504–511.

Sheldon, W. G., et al. (1995). Glaucoma in food-restricted and ad libitum-fed DBA/2NNia mice. *Laboratory Animal Science*, *45*(5), 508–518.

Shimada, Y., et al. (2003). Genetic susceptibility to thymic lymphomas and K-ras gene mutation in mice after exposure to X-rays and N-ethyl-N-nitrosourea. *International Journal of Radiation Biology*, *79*(6), 423–430.

Shimokata, K., et al. (1977). Affinity of Sendai virus for the inner ear of mice. *Infection and Immunity*, *16*(2), 706–708.

Shull, G. H. (1912). "PHENOTYPE" and "CLONE". *Science*, *35*(892), 182–183.

Slattum, M. M., et al. (1998). Progressive necrosing dermatitis of the pinna in outbred mice: An institutional survey. *Laboratory Animal Science*, *48*(1), 95–98.

Smith, R. S., Roderick, T. H., & Sundberg, J. P. (1994). Microphthalmia and associated abnormalities in inbred black mice. *Laboratory Animal Science*, *44*(6), 551–560.

Son, W. C., & Gopinath, C. (2004). Early occurrence of spontaneous tumors in CD-1 mice and Sprague-Dawley rats. *Toxicologic Pathology*, *32*(4), 371–374.

Southard, T., & Brayton, C., (2010). *Spontaneous unilateral brainstem infarction in Swiss mice*. Vet Pathol, In press.

Stoye, J. P., & Coffin, J. M. (1988). Polymorphism of murine endogenous proviruses revealed by using virus class-specific oligonucleotide probes. *Journal of Virology*, *62*(1), 168–175.

Stoye, J. P., Moroni, C., & Coffin, J. M. (1991). Virological events leading to spontaneous AKR thymomas. *Journal of Virology*, *65*(3), 1273–1285.

Sun, J., et al. (2008). The C3H/HeJ mouse and DEBR rat models for alopecia areata: Review of preclinical drug screening approaches and results. *Experimental Dermatology*, *17*(10), 793–805.

Sundberg, B., et al. (2009). A data capture tool for mouse pathology phenotyping. *Veterinary Pathology*, *46*(6), 1230–1240.

Sundberg, J. P., Sundberg, B. A., & Schofield, P. (2008). Integrating mouse anatomy and pathology ontologies into a phenotyping database: Tools for data capture and training. *Mammalian Genome*, *19*(6), 413–419.

Taddesse-Heath, L., et al. (2000). Lymphomas and high-level expression of murine leukemia viruses in CFW mice. *Journal of Virology*, *74*(15), 6832–6837.

Tang, J. C., et al. (1998). Clonality of lymphomas at multiple sites in SJL mice. *Laboratory Investigation*, *78*(2), 205–212.

Tang, J. C., et al. (1998). Progression of spontaneous lymphomas in SJL mice: Monitoring in vivo clonal evolution with molecular markers in sequential splenic samples. *Laboratory Investigation*, *78*(11), 1459–1466.

Taradach, C., & Greaves, P. (1984). Spontaneous eye lesions in laboratory animals: Incidence in relation to age. *Critical Reviews in Toxicology*, *12*(2), 121–147.

Tardieu, M., et al. (1982). Ependymitis, leukoencephalitis, hydrocephalus, and thrombotic vasculitis following chronic infection by mouse hepatitis virus 3 (MHV 3). *Acta Neuropathologica (Berlin)*, *58*(3), 168–176.

Thaete, L. G., Nesbitt, M. N., & Malkinson, A. M. (1991). Lung adenoma structure among inbred strains of mice: The pulmonary adenoma histologic type (Pah) genes. *Cancer Letters*, *61*(1), 15–20.

Thornton, V. B., et al. (2003). Inoculation of *Staphylococcus xylosus* in SJL/J mice to determine pathogenicity. *Contemporary Topics in Laboratory Animal Science*, *42*(4), 49–52.

Tillmann, T., Kamino, K., & Mohr, U. (2000). Incidence and spectrum of spontaneous neoplasms in male and female CBA/J mice. *Experimental and Toxicologic Pathology*, *52*(3), 221–225.

Tsunoda, I., et al. (1996). A comparative study of acute and chronic diseases induced by two subgroups of Theiler's murine encephalomyelitis virus. *Acta Neuropathologica (Berlin)*, *91*(6), 595–602.

Tsunoda, I., et al. (1997). Hydrocephalus in mice infected with a Theiler's murine encephalomyelitis virus variant. *Journal of Neuropathology & Experimental Neurology*, *56*(12), 1302–1313.

Turner, J. G., et al. (2005). Hearing in laboratory animals: Strain differences and nonauditory effects of noise. *Comparative Medicine*, *55*(1), 12–23.

Turrin, N. P. (2008). Central nervous system Toll-like receptor expression in response to Theiler's murine encephalomyelitis virus-induced demyelination disease in resistant and susceptible mouse strains. *Virology Journal*, *5*, 154.

Utsuyama, M., & Hirokawa, K. (2003). Radiation-induced-thymic lymphoma occurs in young, but not in old mice. *Experimental and Molecular Pathology*, *74*(3), 319–325.

Vahle, J., et al. (2009). The international nomenclature project: An update. *Toxicologic Pathology*, *37*(5), 694–697.

Vainzof, M., et al. (2008). Animal models for genetic neuromuscular diseases. *Journal of Molecular Neuroscience*, *34*(3), 241.

Van Loo, P. L., et al. (2001). Modulation of aggression in male mice: Influence of group size and cage size. *Physiology & Behavior*, *72*(5), 675–683.

Van Winkle, T. J., & Balk, M. W. (1986). Spontaneous corneal opacities in laboratory mice. *Laboratory Animal Science*, *36*(3), 248–255.

Verhagen, C., et al. (1995). Spontaneous development of corneal crystalline deposits in MRL/Mp mice. *Investigative Ophthalmology & Visual Science*, *36*(2), 454–461.

Vieira, A. C., et al. (2009). Chloral hydrate anesthesia and lens opacification in mice. *Current Eye Research*, *34*(5), 355–359.

Wahlsten, D. (1982). Deficiency of corpus callosum varies with strain and supplier of the mice. *Brain Research*, *239*(2), 329–347.

Wahlsten, D., Bishop, K. M., & Ozaki, H. S. (2006). Recombinant inbreeding in mice reveals thresholds in embryonic corpus callosum development. *Genes, Brain and Behavior*, *5*(2), 170–188.

Wahlsten, D., Metten, P., & Crabbe, J. C. (2003). Survey of 21 inbred mouse strains in two laboratories reveals that BTBR T/+ tf/tf has severely reduced hippocampal commissure and absent corpus callosum. *Brain Research*, *971*(1), 47–54.

Wakamatsu, N., et al. (2007). Models of human lung cancer. *Toxicologic Pathology*, *35*(1), 75–80.

Ward, J. M., et al. (1994). Chronic active hepatitis and associated liver tumors in mice caused by a persistent bacterial infection with a novel Helicobacter species. *Journal of the National Cancer Institute*, *86*(16), 1222–1227.

Ward, J. M., et al. (2000). Pathology of mice commonly used in genetic engineering (C57BL/6; 129; B6;129; FVB). In J. M. Ward, R. R. Maronpot, & J. P. Sundberg (Ed.), *Pathology of genetically engineered mice* (pp. 161–179). Ames, IA: Iowa State University Press (Blackwell Publishing).

Ward, J. M. (2006). Lymphomas and leukemias in mice. *Experimental and Toxicologic Pathology*, *57*(5–6), 377.

Weiser, K., et al. (2007). Retroviral insertions in the VISION database identify molecular pathways in mouse lymphoid leukemia and lymphoma. *Mammalian Genome*, *18*(10), 709.

Wenzel, K., et al. (2007). Dysfunction of dysferlin-deficient hearts. *Journal of Molecular Medicine*, *85*(11), 1203.

West, J. D., & Fisher, G. (1986). Further experience of the mouse dominant cataract mutation test from an experiment with ethylnitrosourea. *Mutation Research*, *164*(2), 127–136.

Willott, J. F., et al. (1995). Genetics of age-related hearing loss in mice. II. Strain differences and effects of caloric restriction on cochlear pathology and evoked response thresholds. *Hearing Research*, *88*(1–2), 143–155.

Willott, J. F., et al. (2003). Acoustic startle and prepulse inhibition in 40 inbred strains of mice. *Behavioral Neuroscience*, *117*(4), 716–727.

Willott, J. F., Bross, L. S., & McFadden, S. L. (1994). Morphology of the cochlear nucleus in CBA/J mice with chronic, severe sensorineural cochlear pathology induced during adulthood. *Hearing Research*, *74*(1–2), 1–21.

Willott, J. F., & Erway, L. C. (1998). Genetics of age-related hearing loss in mice. IV. Cochlear pathology and hearing loss in 25 BXD recombinant inbred mouse strains. *Hearing Research*, *119*(1–2), 27–36.

Winge, O. (1958). Wilhelm Johannsen: The creator of the terms gene, genotype, phenotype and pure line. *Journal of Heredity*, *49*(2), 83–88.

Won, Y. S., et al. (2002). Identification of *Staphylococcus xylosus* isolated from C57BL/6 J-Nos2(tm1Lau) mice with dermatitis. *Microbiology and Immunology*, *46*(9), 629–632.

Wong, A. A., & Brown, R. E. (2006). Visual detection, pattern discrimination and visual acuity in 14 strains of mice. *Genes, Brain & Behavior*, *5*(5), 389–403.

Wong, A. A., & Brown, R. E. (2007). Age-related changes in visual acuity, learning and memory in C57BL/6 J and DBA/2 J mice. *Neurobiology of Aging*, *28*(10), 1577–1593.

Woyciechowska, J. L., et al. (1984). Acute and subacute demyelination induced by mouse hepatitis virus strain A59 in C3H mice. *Journal of Experimental Pathology*, *1*(4), 295–306.

Yamate, J., et al. (1987). Observations on soft tissue calcification in DBA/2NCrj mice in comparison with CRJ:CD-1 mice. *Laboratory Animals*, *21*(4), 289–298.

Yang, G., et al. (1997). C57BL/6 strain is most susceptible to cerebral ischemia following bilateral common carotid occlusion among seven mouse strains: Selective neuronal death in the murine transient forebrain ischemia. *Brain Research*, *752*(1–2), 209–218.

Yonekura, I., et al. (2004). A model of global cerebral ischemia in C57 BL/6 mice. *Journal of Cerebral Blood Flow & Metabolism*, *24*(2), 151–158.

Zdanowicz, M. M., et al. (1995). High protein diet has beneficial effects in murine muscular dystrophy. *Journal of Nutrition*, *125*(5), 1150–1158.

Zeiss, C. J., Neal, J., & Johnson, E. A. (2004). Caspase-3 in postnatal retinal development and degeneration. *Investigative Ophthalmology & Visual Science*, *45*(3), 964–970.

Zygourakis, C. C., & Rosen, G. D. (2003). Quantitative trait loci modulate ventricular size in the mouse brain. *The Journal of Comparative Neurology*, *461*(3), 362–369.

Chapter 4
The Informatics of High-Throughput Mouse Phenotyping: EUMODIC and Beyond

John M. Hancock and Hilary Gates

Abstract Now that not only the mouse genome sequence but also the ability to carry out high throughput manipulation of mouse ES cells is in place, projects are underway to understand the functions of individual mouse genes in a systematic manner. Central to this will be the systematic analysis of the phenotypes of mutant mouse lines. EUMODIC, the first large-scale project to screen mouse knockout lines for disease-related phenotypes is underway and experience shows the necessity of a well-organised bioinformatics infrastructure for the capture, analysis and dissemination of the data emerging from such projects. Here we discuss the fundamental requirements for such a bioinformatics infrastructure, progress so far and the developments that will be required in future.

4.1 Introduction

The mouse is the most powerful model organism for understanding the organismal malfunctions that lead to disease. This is because of its close evolutionary relationship to humans, broad genetics toolkit and relatively low cost (Rosenthal & Brown, 2007). In order to fully understand how the phenotypes that contribute to disease arise, we need to identify the genes which, when mutated, give rise to these phenotypes. With this knowledge, we will be able to identify pathways involved in producing disease phenotypes and understand how these phenotypes arise and what interventions might allow us to ameliorate or prevent these phenotypes (Brown, Hancock, & Gates, 2006; Brown, Wurst, Kuhn, & Hancock, 2009).

As a first step towards realising these goals, a number of projects have been established to knock out each of the protein-coding genes in the mouse genome. There are four such projects: EUCOMM (European Conditional Mouse Mutagenesis) (Friedel, Seisenberger, Kaloff, & Wurst, 2007), KOMP (Knock-Out Mouse Project; http://www.nih.gov/science/models/mouse/knockout/index.html), NorCOMM (North American Conditional Mouse Mutagenesis project;

J.M. Hancock (✉)
MRC Mammalian Genetics Unit, Harwell, UK
e-mail: j.hancock@har.mrc.ac.uk

C. Brakebusch, T. Pihlajaniemi (eds.), *Mouse as a Model Organism*,
DOI 10.1007/978-94-007-0750-4_4,

http://www.norcomm.org/) and the TIGM (Texas A&M Institute for Genomic Medicine; http://www.tigm.org/) initiative. Collectively these make up the International Knockout Mouse Consortium (IKMC; http://www.knockoutmouse.org/) (Collins, Rossant, & Wurst, 2007) which aims to provide a resource of ES cell lines providing a knockout of each mouse gene.

While the existence of these ES cells will of themselves be massively useful to the research community, for them to reach the widest possible audience more information on the phenotypes of the individual knockout mice will be needed. The strategy being adopted to provide this information is to carry out broad based screening on moderately large cohorts (7–10 males and females) of mice generated from these ES cells. A simple set of screens will be used that provide a high-level insight into which body systems are affected and how these phenotypes relate to major human diseases. In Europe, this strategy has been developed initially by the EUMORPHIA consortium (http://www.eumorphia.org), which developed a set of standardized phenotyping protocols known as EMPReSS (Brown, Chambon, & de Angelis, 2005) and subsequently by the EUMODIC (EUropean MOuse DIsease Clinic) consortium (http://www.eumodic.org). The EUMORPHIA consortium gathered together expert groups from 19 partner institutions to develop and test 150 protocols (standard operating procedures; SOPs). These were collected in the EMPReSS database (http://empress.har.mrc.ac.uk/) (Brown et al., 2005; Green et al., 2005; Mallon, Blake, & Hancock, 2008). EUMODIC builds on the foundations built by EUMORPHIA to carry out systematic screening of up to 650 mouse lines, predominantly derived from ES cells from the EUCOMM project, using a subset of these standardized protocols. Currently moves are afoot to build on the EUMODIC project to develop an international project to phenotype knockout mice representing all the genes knocked out by the IKMC efforts. This represents a huge international effort totalling around 20,000 genes and is being coordinated via a new consortium, the International Mouse Phenotyping Consortium (IMPC). The development of the EMPReSS protocol set and the subsequent logistical issues that have arisen during the implementation of EUMODIC throw into relief the need for a sophisticated bioinformatic infrastructure to collect the information produced in projects of this kind and to make it accessible to the wider mouse and disease research communities in as accessible a manner as possible. In the rest of this article we will describe the requirements, as we see them, for the bioinformatics of systematic mouse phenotyping, progress so far, and what needs to be done in future to provide a truly integrated set of data that will be of broad use to the scientific community.

4.2 Phenotyping Informatics

4.2.1 What Do We Need?

If we consider the various elements of a multi-centre high throughput phenotyping experiment we can compile a list of desiderata for a bioinformatics system that can handle the necessary information. This would consist of:

- *A database of the protocols to be used to carry out the phenotyping.* This should include protocols written in sufficient detail to allow their accurate reproduction by different researchers at different institutions and interpretation of the results by researchers. It should also include information on the measurements to be recorded (termed parameters within EUMODIC) and the units in which they are to be expressed. This is required to ensure consistency of data collection and is also useful for analysis and representation of the data, as will be described later in this article. An additional useful feature is a description of the reasonable limits for parameters. At a naïve level this can be of the form "cannot be less than zero" for a weight, but more useful limits might be used to discriminate falsely entered data into a data capture system.
- *Data capture software.* The experimenters carrying out the project will need access to a system to allow them to capture data. This might form part of a local Laboratory Information Management System (LIMS) if experimenters are in a large mouse clinic, or it might be a stand-alone application specifically designed for the task.
- *A database for phenotype data.* Although data could be made available in crude form by uploading tables of results, e.g. in Excel format, the processing, standardization and analysis of data are made much more powerful by using a relational database structure. Such a database could either be centralized, i.e. held at one site, and receive data from numerous phenotyping centres, or each site could hold its own database. This latter solution gives rise to problems of standardization and data accessibility. Important features of such a database should be that it facilitates quality control of incoming data, is frequently updated, and is easily accessible.
- *A mechanism for transferring data from the data capture software to the database.* There are a number of options for this. One is that the data capture software is part of the phenotype database system and is made accessible via the web, allowing direct input of data into the system. Such a system has a number of problems, such as entering data into a system without adequate quality control and problems arising from loss of access to the central server, although technical solutions to these problems can be envisaged. In cases where the data capture software is situated locally, e.g. as part of a LIM system, a different solution is required whereby data are transferred to the phenotype database using a standard file format.
- *A formalism for representing the data to allow searching.* Raw phenotype data are difficult to interpret and search. For example, if one is looking for genes that cause elevated levels of circulating blood glucose, one needs to know the normal level of circulating blood glucose for a given genetic background and sex. To make searching the data easier it is preferable to annotate the data so that a term such as "elevated circulating blood glucose" can be clicked on and mouse lines matching that term identified directly. For large data sets, and for linking results from different data sets, it is important that the terminologies used should be standard. Because of this requirement, and to facilitate linking of similar phenotype terms, computational structures known as ontologies are commonly used to represent a wide variety of biological information, including phenotypes. Using standard nomenclatures is also important as many phenotyping procedures

generate qualitative, rather than quantitative, data which need to be encoded lexically.

- *A dataset of control phenotypic data.* In order to annotate raw phenotype data from mutant mouse lines it is necessary to have a robust data set which represents the control values produced by the standard procedures applied to mice of the appropriate genetic background and gender. This should provide the basis for statistical analysis of the data to identify significantly deviant phenotypes.
- *Search modalities.* One of the most difficult issues bioinformaticians face is how to present large and/or complex data sets in a way that is intuitive to new users.
- *Standards and Openness.* Any large data set is only valuable to communities it addresses to the extent that it is readily accessible. Genome sequencing has shown the valuable of freely accessible data sets that can be made use of for unforeseen applications. To make data accessible two things are needed: a community commitment to open data availability, and standard formats for making the data transferable and comprehensible. The use of standard ontologies is part of this, but other, internationally agreed standards are also required so that data extracted computationally from databases can be readily and accurately interpreted by its users. Finally, databases need to make data accessible in ways that go beyond user-friendly interfaces for individual users by providing computational access to databases to allow downstream analysis of the data.

In the following section we will review the current state of progress towards these requirements.

4.2.2 State of the Art

4.2.2.1 Protocol Data

The need for a database of phenotyping protocols was recognized early in the development of the EUMORPHIA, which established the EMPReSS database (http://empress.har.mrc.ac.uk/) (Green et al., 2005; Mallon et al., 2008). This database contained protocols broken down into 12 basic headings and represented in a simple custom XML format known as SOPML. Subsequent developments within EUMODIC have resulted in the addition of two important elements to these protocols: information on the data output from the protocol (name, ID, units) (the SOP parameters) and an ontology-based tag which describes the types of phenotypic entity measured by each parameter. Parameters are additionally subdivided into two types: directly measured parameters and derived parameters which involve the arithmetical combination of two or more directly measured parameters. The problem of storing phenotyping protocols is recognized as an important facet of the open accessibility and exchange of phenotype data (Mouse Phenotype Database Integration Consortium, 2007), as data cannot readily be interpreted if the methods used to generate them are not known. In this way phenotype data are radically different from genome data, but more similar, for example, to microarray gene expression data. The XML format originally used to describe protocols within

EMPReSS was relatively simple. Standards for the representation of phenotyping procedures are therefore being developed further within the broader framework of MIMPP (Minimum Information for Mouse Phenotyping Procedures) (Taylor et al., 2008), leading to a more sophisticated XML schema for protocols, PPXML (http://www.interphenome.org/ppxml/ppml_v1_3.html).

4.2.2.2 Data Capture

EUMODIC presently represents the best example of distributed capture of phenotype data. This network carries out phenotyping in four centres (MRC Harwell and the Wellcome Trust Sanger Institute in the UK, Institut Clinique de la Souris, France and the German Mouse Clinic, Germany), all of which are established, large mouse clinics with their own LIM systems which have been developed in different ways and to different standards. EUMODIC has therefore not performed any centralized development relevant to first-line data capture, and indeed this is largely untrodden territory. It is clear, however, that if the international phenotyping is to be ramped up there will be a need for many new, large-scale phenotyping centres with their own LIMS and for smaller data capture tools that can be used by individual laboratories that wish to contribute to the worldwide phenotyping effort. A project that is addressing this in part is InfraFrontier (http://www.infrafrontier.eu/), a European infrastucture project that aims to investigate, among other things, the LIM systems currently in use internationally within mouse laboratories and come up with recommendations for the likely data capture requirements of newly established mouse phenotyping centres.

4.2.2.3 Phenotype Databases

Although the Mouse Genome Database (Blake, Bult, Eppig, Kadin, & Richardson, 2009) contains a large amount of information on mouse phenotypes, this is information collected from the literature by curators and does not include raw phenotyping data. The first systematic collection of raw phenotyping data has been made by the Mouse Phenome Project (Bogue & Grubb, 2004; Grubb, Maddatu, Bult, & Bogue, 2009), which collects data generated in individual labs on a large number of inbred mouse lines. The first systematic phenotyping of mouse gene knockouts was carried out by Deltagen Incorporated and Lexicon Genetics in the US and databases of these results of these screens were constructed. These datasets are now available from the Jackson Laboratory web site (http://www.informatics.jax.org/external/ko/). EuroPhenome (http://www.europhenome.org) (Morgan et al., 2010) was established during the EUMORPHIA project to play a parallel role to Mouse Phenome Database (MPD) (Grubb et al., 2009) for the validated data on four inbred lines produced by the project. Subsequently it was expanded to incorporate data generated from mutant lines and their controls by the EUMODIC consortium, where it serves as the central repository for the consortium's phenotype data. EuroPhenome is an open source software project based around a MySQL relational database. The EuroPhenome web portal is actually underlain by three linked databases: EuroPhenome itself, the EMPReSS database, and OATH, a database of ontology annotations.

4.2.2.4 Data Transfer

Whereas MPD supports submission of phenotype data as spreadsheet or text files (see http://phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/dataguide) EuroPhenome has taken a more automated approach. In this approach the phenotyping centres export their data into standard XML formats which is then accessed via their local FTP sites by EuroPhenome (Morgan et al., 2010). These XML files describe both the genetic makeup and other features of individual mice or cohorts of mice and the phenotyping data associated with those mice. By providing data in a standard format, much of the processing, including file validation, can be carried out automatically, reducing (but not eliminating) the potential for human error. This approach has the added benefit that the phenotyping centres do not have to capture the data twice, or format data files manually. It is also relatively scalable, because it does not require as much manual intervention at the database end and so provides a potential model for any larger scale effort in future, such as the IMPC.

4.2.2.5 Data Representation

Ontologies have been used for the representation of biological data for over 10 years, starting with the initial development of the Gene Ontology (Ashburner et al., 2000). The Mouse Genome Informatics group at the Jackson Laboratory developed the Mammalian Phenotype ontology (MP) (Smith, Goldsmith, & Eppig, 2005) as a standard structure for describing mouse, and subsequently rat, phenotypes. MP allows the identification of abnormal phenotypes but not the quantitative description of phenotype data or the annotation of normal data (this partly reflects differences in the definition of the term "phenotype" which some consider only to include abnormal states of an organism while others consider it to cover normal and abnormal states). An alternative approach to representing phenotype data is to use the EQ approach (Gkoutos, Green, Mallon, Hancock, & Davidson, 2004; 2005). In this, qualities, or properties, are associated with features of an organism such as anatomical parts or aspects of behaviour which themselves are encoded within entity ontologies such as the Mouse Anatomy ontology (Hayamizu, Mangan, Corradi, Kadin, & Ringwald, 2005). The combination of entity terms with quality terms from the quality ontology PATO can represent either the class of phenotype to be measured or the value observed in an individual mouse or group of mice. EuroPhenome uses a combination of these two approaches with statistical analysis to provide a first pass automatic annotation of mouse lines (Beck et al., 2009).

4.2.2.6 Baseline Phenotype Data

As mentioned previously, baseline data for the lines analysed in EUMODIC are stored in EuroPhenome. These are used for automatic, first-pass annotation of mutant data (Beck et al., 2009). There is an unresolved difficulty with this approach, which is determining which mice should be used as a control cohort. In the extreme case it can be argued that only those mice which have been through the same phenotyping tests on the same day (and have the same genetic background) can

appropriately act as controls. However this gives rise to significant logistical issues and some centres within EUMODIC use intermittent measurements on a standard background strain to provide a baseline. This baseline can then be used for the comparison of mutant strains with problems arising only if a significant shift in this baseline is observed.

4.2.2.7 Search Interfaces and Analysis Tools

Raw phenotype data is a relatively new class of data and in many ways it is more complex than the data that bioinformaticians are used to dealing with. Genome data, for example, is essentially linear in nature and has a fairly natural representation that is immediately obvious to most users. This is not the case for phenotype data. One natural representation made use of in EuroPhenome is to make use of the MP ontology tree as a means to find phenodeviants by starting from the top level, identifying a general class of phenotype of interest, and then burrowing down to more detailed phenotypic descriptions. Alternatively, text searches can be used to identify strains annotated with features of interest, but this approach suffers from the potential for missing annotations because the search terms do not match the ontology terms. A third approach is to produce a heat-map style of visualization, familiar to those who analyse gene expression data, which immediately identifies phenotypic areas with significantly deviant phenotypes. These approaches are illustrated in Fig. 4.1.

EuroPhenome currently has few data analysis tools but some interesting examples of useful tools can be found on the MPD site (http://phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/corrcenter). These allow correlation of strain phenotypes or phenotype measurements across strains and plotting of possible sources of phenotypic deviation on the reference mouse genome. In future more sophisticated analysis will also become possible, such as mapping phenotype-causing genes onto biological pathways or networks, and displaying phenotypes in association with anatomical images and gene expression data to relate gene expression to phenotype. These are early days for the visualization and analysis of phenotype data and it is likely that new modalities will emerge in the coming years.

4.2.2.8 Standards and Openness

Open access to the products of large-scale (“community”) projects in the biosciences has been the dominant paradigm since the publication of the Bermuda Principles in 1996 (HUGO, 1996). More recently, moves have started to encourage the open accessibility of a wider range of data on the basis that they are (predominantly) publicly or charity funded and that free access to data facilitates the progress of scientific understanding (Schofield et al., 2009). However to make best use of freely available data, researchers need, apparently paradoxically, to show greater discipline in how they prepare that data for sharing, making use of widely accepted standards for data representation and exchange so that other researchers and computer systems can interpret what they wish to share. This issue was identified in the phenotype data domain by the Mouse Phenotype Database Integration Consortium (informally known as Interphenome) (Mouse Phenotype Database Integration Consortium,

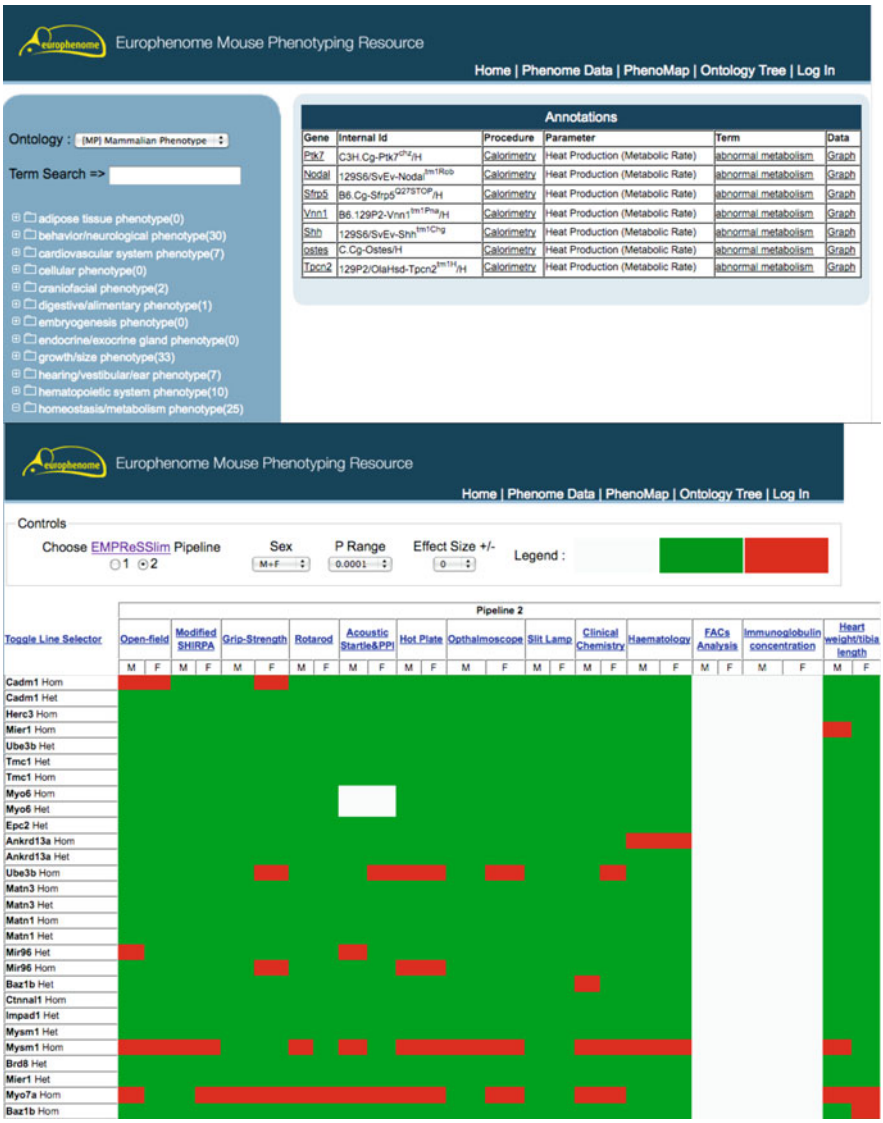

Fig. 4.1 Examples of graphical views used to identify phenodeviants within the EuroPhenome database. *Top*: Alleles annotated as showing significantly abnormal phenotypes are identified by clicking on the relevant MP ontology tree in the tree structure on the left hand side of the browser. The table allows the user to access the data that underlies the annotation. *Bottom*: A heat map view of phenotype tests showing significant deviation from normality. A *red* cell indicates that at least one parameter in the test (shown on the *x*-axis) shows significant deviation from normal for a given gene. *P*-value and Effect Size cut-offs can be adjusted to limit the number of positives as the user desires

2007) which identified three main areas in which progress was required: data representation using ontologies, the formal representation of phenotyping protocols, and the need for a standard exchange format for phenotype data. Some progress has been made in all of these areas since the original Interphenome publication. A start has been made to the representation of all MP terms using the EQ formalism, a development necessary to map the atomistic descriptions in EQ to the more human-friendly descriptions of MP. EuroPhenome uses these mappings to generate MP annotations. The MIMPP project has been developed as a framework for developing a standard for describing phenotyping procedures, giving rise to an XML schema that is an advance on the original used in the EMPReSS database. Finally recent discussions including MRC Harwell, the RIKEN Bioresource Center, Japan, the Jackson Laboratory and the Toronto Phenogenomics Centre, under the auspices of the EU-funded CASIMIR project (Coordination and sustainability of Mouse Informatics Resources) (Hancock et al., 2008), have developed a draft format for the exchange of so-called shallow phenotype data which it is intended to test in 2010. Shallow phenotype data essentially consists of gene identifiers associated with ontology terms, with some additional qualifying metadata. A fuller format for exchange of complete datasets is likely to follow. These developments will facilitate the free interchange of mouse phenotype data and the construction of integrating portals and databases to this information. A final issue that has arisen recently is the need for agreement on the conditions under which data are shared (are they to be put into the public domain or subject to some form of licensing?) and how to cite items of phenotype data accessed from databases. This latter area in particular is one in which active development is likely to take place in the coming years.

4.2.3 Future Perspectives

Approaches to mouse phenotype bioinformatics have evolved in parallel with the evolution of experimental approaches to mouse phenotyping. With the likely transition of systematic mouse phenotyping into a global activity there will also need to be a step change in the bioinformatics approaches being taken. Fortunately considerable groundwork has been laid and the directions are generally clear, although a lot of detail remains to be put in place. Areas of particular emphasis for the future include developing more sophisticated modes of data representation, particularly linking mouse phenotype data to human disease data and phenotype data from other organisms (Hancock et al., 2009); the development of technological solutions to the sharing of phenotype data at both the shallow and deep levels and resolution of the question whether all of this data should be consolidated in a central database; and the development of more sophisticated approaches to displaying and analyzing phenotype data in ways that will best add value to the mass of data that is likely to emerge over the next decade.

Acknowledgements We thank the many contributors to the development of the ideas described here including the developers of the Mouse Genome Database, Mouse Phenome Database and the partners in EUMORPHIA and EUMODIC. We thank the UK Medical Research Council and

the European Commission for financial support. EUMORPHIA and EUMODIC were funded by the European Commission under contract numbers QLG2-CT-2002-00930 and LSHG-CT-2006-037188.

References

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., et al. (2000). Gene ontology: Tool for the unification of biology. The Gene Ontology Consortium. *Nature Genetics*, *25*(1), 25–29.

Beck, T., Morgan, H., Blake, A., Wells, S., Hancock, J. M., & Mallon, A. -M. (2009). Practical application of ontologies to annotate and analyse large scale raw mouse phenotype data. *BMC Bioinformatics*, *10*(Suppl 5), S2.

Blake, J. A., Bult, C. J., Eppig, J. T., Kadin, J. A., & Richardson, J. E. (2009). The mouse genome database Genotypes::Phenotypes. Nucleic Acids Research, 37(Database issue), D712–719.

Bogue, M. A., & Grubb, S. C. (2004). The mouse phenome project. *Genetica*, *122*(1), 71–74.

Brown, S. D., Chambon, P., & de Angelis, M. H. (2005). EMPReSS: Standardized phenotype screens for functional annotation of the mouse genome. *Nature Genetics*, *37*(11), 1155.

Brown, S. D. M., Hancock, J. M., & Gates, H. (2006). Understanding mammalian genetic systems: The challenge of phenotyping in the mouse. *PLoS Genetics*, *2*(8), e118.

Brown, S. D., Wurst, W., Kuhn, R., & Hancock, J. (2009). The functional annotation of mammalian genomes: The challenge of phenotyping. *Annual Review of Genetics*, *43*, 305–333.

Collins, F. S., Rossant, J., & Wurst, W. (2007). A mouse for all reasons. *Cell*, *128*(1), 9–13.

Friedel, R. H., Seisenberger, C., Kaloff, C., & Wurst, W. (2007). EUCOMM–the European conditional mouse mutagenesis program. *Briefings in Functional Genomics Proteomic*, *6*(3), 180–185.

Gkoutos, G. V., Green, E. C. J., Mallon, A. -M., Hancock, J. M., & Davidson, D. (2004). Building mouse phenotype ontologies. *Pacific Symposium on Biocomputing*, *9*, 178–189.

Gkoutos, G. V., Green, E. C. J., Mallon, A. -M., Hancock, J. M., & Davidson, D. (2005). Using ontologies to describe mouse phenotypes. *Genome Biology*, *6*(1), R8.

Green, E. C. J., Gkoutos, G. V., Lad, H. V., Blake, A., Weekes, J., & Hancock, J. M. (2005). EMPReSS: European mouse phenotyping resource for standardised screens. *Bioinformatics*, *21*(11), 2930–2931.

Grubb, S. C., Maddatu, T. P., Bult, C. J., & Bogue, M. A. (2009). Mouse phenome database. *Nucleic Acids Res*, *37*(Database issue), D720–730.

HUGO Summary of Principles Agreed at the First International Strategy Meeting on Human Genome Sequencing Bermuda, 25–28 February 1996. 1996:available at www.ornl.gov/sci/techresources/Human_Genome/research/bermuda.shtml.

Hancock, J. M., Mallon, A. M., Beck, T., Gkoutos, G. V., Mungall, C., & Schofield, P. N. (2009). Mouse, man, and meaning: Bridging the semantics of mouse phenotype and human disease. *Mammalian Genome*, *20*(8), 457–461.

Hancock, J. M., Schofield, P. N., Chandras, C., Zouberakis, M., Aidinis, V., Smedley, D., et al. (2008). Consortium: CASIMIR: Coordination and Sustainability of International Mouse Informatics Resources. In: *8th IEEE International Conference on Bioinformatics and Bioengineering: Oct 8–10 2008*, Athens, Greece: IEEE, pp. 382–387.

Hayamizu, T. F., Mangan, M., Corradi, J. P., Kadin, J. A., & Ringwald, M. (2005). The adult mouse anatomical dictionary: A tool for annotating and integrating data. *Genome Biology*, *6*, R29.

Mallon, A. M., Blake, A., & Hancock, J. M. (2008). EuroPhenome and EMPReSS: Online mouse phenotyping resource. *Nucleic Acids Research*, *36*(Database issue), D715–718.

Morgan, H., Beck, T., Blake, A., Gates, H., Adams, N., Debouzy, G., et al. (2010). EuroPhenome: A repository for high-throughput mouse phenotyping data. *Nucleic Acids Research*, *38*(Database Issue), D577–D585.

Mouse Phenotype Database Integration Consortium (2007). Integration of mouse phenome data resources. *Genome Biology*, *18*, 157–163.

Rosenthal, N., & Brown, S. (2007). The mouse ascending: Perspectives for human-disease models. *Nature Cell Biology*, *9*(9), 993–999.

Schofield, P. N., Bubela, T., Weaver, T., Portilla, L., Brown, S. D., Hancock, J. M., Hrabede Angelis M., Rosenthal N., et al. (2009). Post-publication sharing of data and tools. *Nature*, *461*, 171–173.

Smith, C. L., Goldsmith, C. A., & Eppig, J. T. (2005). The Mammalian Phenotype Ontology as a tool for annotating, analyzing and comparing phenotypic information. *Genome Biology*, *6*(1), R7.

Taylor, C. F., Field, D., Sansone, S. A., Aerts, J., Apweiler, R., Ashburner, M., et al. (2008). Promoting coherent minimum reporting guidelines for biological and biomedical investigations: The MIBBI project. *Nature biotechnology*, *26*(8), 889–896.

Chapter 5
Experimental Tumour Models in Mice

Ritva Heljasvaara and Taina Pihlajaniemi

Abstract The complex multistage processes of tumour initiation, progression and metastasis challenge the methods that are used in basic cancer biology research and anticancer drug development. Experimental tumour modelling in mice provides means for observing tumour development, identifying target molecules and pathways and designing and testing novel strategies for diagnosing and treating cancer in a manner that is not possible in in vitro systems or in human patients. To gain maximal benefit from the use of mouse tumour models one should be aware of the possibilities and limitations of each approach, and pay careful attention to selection of the model and planning of the experiments. We aim in this review to give the reader some basic information on experimental mouse tumour models that have evolved from simple chemical treatments to extremely complex genetic models, and to discuss their advantages and disadvantages. We discuss some species-specific differences between mice and humans, and also between the inbred mouse strains, that can affect the various processes of tumorigenesis. Finally, we try with a few examples of cancer studies involving the p53 and retinoblastoma tumour suppressors, and the extracellular matrix protein collagen XVIII and its antiangiogenic endostatin fragment to illustrate the importance of evaluating data from various tumour models in order to achieve a proper understanding of the function of a given molecule or pathway in tumour development.

5.1 Introduction

Tumour initiation, progression and metastatic dissemination to distant sites are complex multifactorial processes. Besides the multiple genetic alterations that occur in a stepwise manner in cancer genes and their modifier genes during malignant transformation, a number of other factors affect primary tumour growth and metastasis (Hanahan & Weinberg, 2000; Hahn & Weinberg, 2002). These include several

R. Heljasvaara (✉)
Oulu Center for Cell-Matrix Research, Biocenter Oulu, University of Oulu, FIN-90014, Oulu, Finland; Department of Medical Biochemistry and Molecular Biology, Institute of Biomedicine, University of Oulu, FIN-90014, Oulu, Finland
e-mail: ritva.heljasvaara@oulu.fi

C. Brakebusch, T. Pihlajaniemi (eds.), *Mouse as a Model Organism*,
DOI 10.1007/978-94-007-0750-4_5,

epigenetic regulatory mechanisms such as DNA methylation, microRNA regulation and histone modifications (Sharma, Kelly, & Jones, 2010). Cancer progression also involves numerous interactions between transformed cells and their constantly evolving microenvironment, consisting of the extracellular matrix (ECM), vasculature and stromal cells (inflammatory cells and cancer-associated fibroblasts) which produce growth factors, cytokines, inhibitors, proteases and ECM components to affect the ultimate process of tumour growth (Hanahan & Weinberg, 2000; Hahn & Weinberg, 2002). This complexity in tumour development places high requirements on the methods that are used for cancer research.

Tumour modelling in mice has significantly contributed to our current understanding of cancer and its treatment. Existing and novel hypotheses on carcinogenesis are currently being tested in novel in vivo model systems, particularly using genetically modified mice (Frese & Tuveson, 2007). Experimental cancer research can roughly be divided into two categories: basic cancer biology studies which focus on the molecules and mechanisms underlying tumour growth and metastasis, and the development of cancer therapies and their preclinical testing in animals. The latter also involves important pharmacological and toxicological issues and specific practices and criteria for evaluating tumour response, as discussed in detail elsewhere (Bibby, 2004; Schuh, 2004; Suggitt & Bibby, 2005; Talmadge, Singh, Fidler, & Raz, 2007). Nevertheless, for both purposes the choice of relevant tumour models and appropriate experimental conditions is absolutely critical.

5.2 The Mouse as a Model Organism in Cancer Research

In vitro cell and organotypic culture systems are powerful and constantly improving methods for studying many aspects of tumour growth, but they do not allow us to investigate entire physiological systems and their interactions, nor processes such as angiogenesis and metastasis. Therefore in vivo animal models are indispensable for cancer research. The laboratory mouse (*Mus musculus*) is considered the most accurate and accessible model organism for cancer studies for several reasons. First, it has a similar organ system and systemic physiology to humans and shares genetic features with humans. The majority of the human genes have murine counterparts, and the functions of these genes are in most cases closely related. Gene mutations that cause certain diseases in humans often, but not always, cause similar diseases in mice (Paigen, 2003; Peters et al., 2007). Furthermore, mice are small in size and easy to house in the laboratory. They have a relatively short breeding cycle and can produce a large number of offspring. An additional, one unique advantage over other higher laboratory animals is associated with the mouse, namely our ability to manipulate the mouse genome. Thus, transgenic mice can be created to overexpress activated oncogenes, tumour suppressor genes can be inactivated (knock-out models) or selected cancer genes can be mutated in a precise manner to create replicas of the genetic defects that cause cancer in humans (knock-in models). These methodologies have led to a number of key discoveries in cancer pathogenesis and are continuously producing increasingly sophisticated in vivo models for cancer studies (Rangarajan & Weinberg, 2003; Frese & Tuveson, 2007).

There are several important species-specific differences (genetic, molecular, cellular, developmental and metabolic) between humans and mice, however, which can affect tumour development and which it is thus critical to understand in order to design experiments and interpret their results. These differences include the species' susceptibility for developing cancer over their lifetime. Humans have a significantly longer lifespan, are larger in size and have larger numbers of cells undergoing division, so that in theory they have an increased risk of DNA damage which may ultimately lead to cancer. Yet they show markedly lower cancer rates upon ageing than mice. It has been estimated that an accumulation of 4–7 mutations is needed to transform a human epithelial cell, whereas fewer changes are required for murine cells. This can at least partly be explained by differences between the mouse and man in the retinoblastoma (Rb) and p53 pathways and telomere function that control the division and lifespan of cells, as discussed in detail elsewhere (Hanahan & Weinberg, 2000; Hahn & Weinberg, 2002; Rangarajan & Weinberg, 2003).

The tumour spectrum and karyotype also vary between the two species. Laboratory mice tend to develop mesenchymal tumours such as sarcomas and lymphomas, whereas humans are more prone to epithelial carcinomas (Rangarajan & Weinberg, 2003; Anisimov, Ukraintseva, & Yashin, 2005; Maddison & Clarke, 2005) These human epithelial tumours show highly abnormal karyotypic profiles characterized by changes in chromosome number and multiple, non-reciprocal translocations, features that are uncommon in murine tumours (Rangarajan & Weinberg, 2003). The tumours that develop in telomerase-deficient mice in old age show similar translocations, however, and it has been suggested that telomere dysfunction in human cancer cells might also be linked to abnormal cytogenetic profiles (Artandi et al., 2000).

5.3 What Constitutes an Optimal Mouse Model for Cancer Research?

Mouse tumour models have progressed from chemical carcinogen models and transplantable murine tumours to advanced genetic models with increased susceptibility to tumour development. Each of these approaches has its own advantages and limitations, which should be considered when choosing the most appropriate model for a particular question. By paying careful attention to the possibilities of a given model and experimental design (e.g. mouse strain and sex, immunological status of the host, method and site of tumour implantation, selection of endpoints), one can extract the most reliable information from the experiment. As a general rule, an experimental model in basic cancer research should mimic the counterpart human disease in clinical, genetic and histopathological terms, whereas in drug testing it is faithful recapitulation of the targeted neoplastic event that is crucial. Nevertheless, one should always bear in mind that all mouse models are more or less imperfect when it comes to recapitulating human malignancies (Schuh, 2004; Frese & Tuveson, 2007).

One should also consider several practical issues such as convenient monitoring methods, penetration rates and tumour latency periods which all contribute to a manageable mouse cohort and the cost-effectiveness of the model. It should be noted that in rapidly growing tumours with high penetrance, such as genetic tumour models with strong oncogene expression and concomitant loss of a tumour suppressor, the evolution of the microenvironment and acquisition of secondary oncogenic mutations (imperative for neoplastic transformation in human cells) are not necessarily simulated accurately (Frese & Tuveson, 2007).

One extremely important issue is the ethics of the tumour model. According the principle of the 3 R's (replacement, reduction and refinement) (Russel & Burch, 1992), when replacement of a murine model with an alternative in vitro method or lower animal species is not possible, the number of mice can be potentially reduced (without losing important information) by using genetically homogenous inbred mouse strains instead of hybrids (still recognising that humans are not genetically homogenous), and by careful experimental design and controlled conditions. The experiment should also be refined, which ensures that the mice are treated with care, and that the pain, discomfort and stress that the animals experience are minimized. In tumour experimentation this includes limitation of the tumour burden, determination and respect of humane end points, the use of non-invasive in vivo monitoring methods, and the technical competence of the laboratory personnel.

5.4 Transplantable Tumour Models

A major breakthrough in experimental cancer modelling was the development of transplantable rodent tumours (Corbett, 2002). Numerous immortalized cell lines of human and mouse origin are tumorigenic in mice and routinely used in cancer research (see www.sanger.ac.uk/perl/genetics/CGP). These cells are maintained and amplified in culture, and usually implanted by simple subcutaneous (s.c.) injection into the flank of the mouse. Transplantable tumour models have a number of advantages over other experimental mouse models. The immortal cell lines are readily available, easy to maintain and in many cases the specific mutations in cancer genes are known (www.sanger.ac.uk/perl/genetics/CGP). Tumour implantation is fast and simple and does not require any specific expertise. Tumour growth is synchronized and generally rapid, and tumour monitoring by palpation and caliper measurements in the s.c. location is easy (Schuh, 2004; Becher & Holland, 2006). The emerging non-invasive in vivo imaging systems for fluorescent protein or luciferase-tagged tumour cells (also readily available, see www.metamouse.com) represent a major methodological and ethical improvement in transplantation models (Hoffman, 2005). Moreover, transplantation is highly reproducible and a relatively small number of mice is needed which contributes to the cost-effectiveness of this tumour model.

One of the main drawbacks in the use of transplantable tumorigenic cell lines is that they have been passaged for many generations in culture and, due to selection pressure under in vitro conditions, accumulate additional mutations and do

not represent the original tumour in its native state. This means that they may display altered growth characterisitics, protein expression profiles and drug sensitivity, and also altered histomorphology when inoculated into mice. Moreover, implanted tumours model cancer as if it was a disease of a homogenous pool of cells, and lack the morphological and cellular complexity of in vivo tumours.

Quality control is also an important issue in the context of transplanted tumour cells. Many cell lines have undocumented source and passage histories, and subclones in different laboratories may diverge from the parental cell line, causing altered responses when inoculated into mice (Schuh, 2004; Becher & Holland, 2006). This is exemplified by a study of collagen XVIII/endostatin, related to our own work, where implanted murine Lewis lung carcinoma and B16F10 melanoma cells producing antiangiogenic endostatin showed equivalent tumour growth rates and microvessel densities in collagen XVIII/endostatin knock-out and control mice, whereas a variant B16F10 subclone lacking expression of this collagen displayed increased angiogenesis and grew faster in the knock-out mice (Sund et al., 2005). This clearly indicates that tumour growth is determined not just by the genetic defects accumulating in the cancer cells but also by host-derived factors, and that these host-derived effects can sometimes be masked by the proteins produced by the implanted tumour cell. This example also highlights the importance of testing various tumour cell lines, and preferably also other experimental cancer models.

5.4.1 Xenograft Models

The term xenograft refers to the transplantation of cultured human tumour cells or human tumour explants into immunodeficient mice which do not reject the graft. The most common immunodeficient mouse strains used in xenograft models are hairless nude mice (nu/nu), which completely lack the thymus, and SCID (severe combined immunodeficiency) mice, in which the differentiation of haematopoietic stem cells into mature T and B lymphocytes is hindered. These mice have normal innate immune cells such as macrophages and natural killer cells, however, which can affect tumorigenesis and metastatic spread in xenograft systems (Jacob, 2004; Schuh, 2004).

The first successful xenotransplant of cultured human melanoma cells in nude mice was achieved almost 40 years ago (Giovanella, Yim, Stehlin, & Williams, 1972). Today a wide variety malignant human tumour-derived or genetically engineered cell lines are readily available, some backed by an extensive literature and others with reporter gene expression. Xenograft models are generally thought to be of only limited value in basic cancer research, but since human tumour cells with human cellular processes are used in this system, they are particularly suitable for preclinical anticancer drug validation after an in vitro pre-screen with highly sensitive human tumour cell lines such as MCF-7 breast carcinoma, NCI-H460 lung carcinoma and SF-268 glioma (Suggitt & Bibby, 2005).

Unfortunately, there are some gross disadvantages associated with xenograft models. Nude and SCID mice have severe defects in their immune systems which

can either promote or restrict tumour growth or limit their capacity to tolerate various treatments (Schuh, 2004). Xenograft tumours may also show increased response to anticancer agents. In addition, even though human tumour cells can be implanted into immunodeficient mice, the system is not ideal because the tumour microenvironment is still of murine origin and does not allow perfect interactions with neoplastic human cells. This limitation can be partially overcome by using humanized mice, such as NOD/SCID (non-obese diabetic/SCID) or Rag (recombination-activating gene) null mice engrafted with human haematopoietic cells or tissues (Schuh, 2004; Shultz, Ishikawa, & Greiner, 2007).

5.4.2 Syngeneic Models

In a syngeneic model cultured murine tumour cells are implanted into the mouse strain from which the tumour cell originates. This system overcomes the problems of incompatibility between the tumour cell and the murine stroma of the host and the immunodeficiency of the host, but still possesses most of the other limitations, and obviously also the advantages, associated with transplantable tumour models. Thus, a syngeneic microenvironment in the host allows the essential interactions between tumour cell receptors and stromal ligands and the functioning immune system of the host creates a more realistic milieu for tumour development and also facilitates drug testing (Corbett, 2002). On the other hand, murine tumour cells may show profound differences in cellular processes compared with the corresponding human tumour cells, as discussed above, such as the differences in telomere function.

There are numerous C57BL/6-derived tumour cells available from different depositors, but FVB/N-derived tumour cells, for example, are rather unusual. While many of the early-established murine tumour cell lines were carcinogen-induced and others developed naturally in old inbred mice, the later tumorigenic cell lines are often genetically engineered or derived from tumours of genetically modified mice (Corbett, 2002). One example is the widely used C57BL/6-derived B16 melanoma, which can be employed to follow the processes of solid tumour formation and metastatic spread. The B16 melanoma cell line originates from a spontaneous mouse skin melanoma, and after s.c. implantation colonizes the lung, liver and spleen. Intravenous injections of parental B16 cells and subsequent serial in vivo−in vitro selection processes have resulted in different subclones with specific metastatic potentials in the lungs (B16F10), brain (B16B19b, B16B10n) and ovaries (B17-010) (Alvarez, 2002), for example.

5.4.3 Orthotopic Models

Anticancer therapies showing promising responses in preclinical testing with mice often fail in clinical use. This has been the case with collagen XVIII-derived endostatin, for example, which proved extremely efficient in reducing angiogenesis and primary tumour growth in experimental mouse models (Folkman, 2006) but showed

no antitumour activity in human clinical trials using standard response criteria (Kulke et al., 2006; Moschos et al., 2007). One reason behind this disparity is that the conventional s.c. xenograft and syngeneic systems used in preclinical testing do not represent advanced disease. Indeed, the lack of metastasis from the subcutaneous growth site often limits the use of these approaches. This has led to the development of clinically more relevant and accurate cellular and surgical orthotopic models in which human or mouse-derived neoplastic cells or intact small tumour explants are injected or surgically implanted (surgical orthotopic implantation, SOI) into the anatomical site of the primary tumour (Manzotti, Audisio and Pratesi, 1993; Hoffman, 1999; Bibby, 2004; Schuh, 2004). The realistic microenvironment provided in orthotopic models enhances primary tumour growth and enables local invasion and angiogenesis studies to be carried out in a clinically relevant location. Orthotopic tumours also show increased metastatic potential and they often disseminate in a similar manner and to the same distant organs as the primary human tumours. Recent observations indicate that the tumour site used in an experimental model also affects the response to therapeutic agents (Bibby, 2004). Due to the complex surgical techniques, however, which require special technical expertise and enable only a small number of mice to be used per experiment, orthotopic models are employed mainly in basic cancer biology research and are not widely used in anticancer drug development or preclinical testing (Hoffman, 1999).

5.5 Autochthonous Models

An autochthonous tumour is described as an endogenous or *in situ* tumour that develops from the animal's own normal cells. Autochthonous models include spontaneously occurring tumours in mice and cancers induced by chemicals, which will be discussed here in more detail. Physical treatments, e.g. with UV light, asbestos fibres, viruses or their genes, including murine polyoma and simian virus 40 T antigen or human papillomavirus E6/E7 antigen, or with microbes such as *Helicobacter*, can also be used to evoke tumour formation in mice (Suggitt and Bibby, 2005; Frese & Tuveson, 2007; Talmadge, Singh, Fidler, & Raz, 2007). It is generally thought that autochthonous models mimic tumour development more precisely than transplanted tumours. The advantageous properties of these models include an orthotopic growth site, an opportunity to monitor different stages in tumour development from hyperplasia to advanced malignant cancers, and authentic metastasis routes through the blood and lymphatic vessels (Berger, 1999). Autochthonous models have contributed to many basic discoveries in cancer biology and are also of importance as a source of many tumour cell lines used in in vivo transplantation models and in vitro studies (Schuh, 2004), but for several reasons their use in drug development is limited and they are only occasionally used for advanced or phase II drug screening (Berger, 1999). The significant limitations associated with autochthonous models include long tumour latency periods in many models and substantial variability in

tumour incidence and multiplicity, resulting in the need to use a large number of animals (Schuh, 2004; Frese & Tuveson, 2007; Talmadge, Singh, Fidler, & Raz, 2007).

5.5.1 Spontaneous Tumour Models in Mice

As discussed earlier, the spectrum of age-related spontaneous tumours differs considerably between humans and mice, and the genetic background also plays a significant role in dictating the exact tumour spectrum in different mouse strains. The most frequent tumour type in the widely used C57BL/6 strain, for example, is malignant lymphoma/leukaemia, whereas old FVB/N mice often develop lung adenocarcinomas but seldom lymphomas (Mahler, Stokes, Mann, Takaoka & Maronpot, 1996; Paigen, 2003; Anisimov, Ukraintseva, & Yashin, 2005; http://www.informatics.jax.org/external/festing). Additional spontaneous, induced (often with the mutagen ethylnitrosourea, ENU) or genetically engineered mutations in inbred mouse strains can predispose them to new spontaneous tumours in selected organs or tissues, or change the existing strain-specific tumour pattern (DePinho, 2000; Attardi & Donehower, 2005). Such mouse strains can be used as models for studying particular human cancers. A classical example of a spontaneous tumour model is the $Apc^{Min/+}$ mouse, in which an ENU-induced mutation in the adenoma polyposis coli (*Apc*) gene leads to the formation of multiple intestinal adenomas and provides a mouse model for human familiar colon cancer syndromes caused by mutations in the human *APC* gene (Moser, Pitot, & Dove, 1990). In another example, old telomerase-deficient mice that were heterozygous for the p53 tumour suppressor showed a shift in the tumour spectrum from lymphomas and sarcomas to carcinomas of the breast, colon and skin with cytogenetic profiles typical of human carcinomas (Artandi et al., 2000).

5.5.2 Chemical Models

Certain environmental factors such as coal tar, metals and dyes increase tumour incidence in exposed humans. The pioneer study of experimental chemical carcinogenesis was conducted in 1915 by painting rabbits' ears with coal tar, which caused the formation of epithelial skin tumours. It was only much later, however, that the DNA-targeting carcinogenic molecules in the coal tar and other environmental chemicals and their mechanisms of action were identified. Coal tar was shown to contain several polycyclic aromatic hydrocarbons (PAHs), such as dibenzanthracene and benzopyrene, that form covalent adducts with DNA and induce cancer in mouse skin. A wide variety of chemicals can be used to induce cancers in laboratory animals, affecting different organs depending on the administration routes and protocol. Diethylnitrosamine (DEN), for example, can be used to produce tumours in the liver, lung and oesophagus, and 7,12-dimethylbenz[α]anthracene (DMBA) in the skin, mammary gland and colon (Luch, 2005; Loeb & Harris, 2008).

The two-stage model of mouse skin carcinogenesis on exposure to a specific initiation-promotion regimen is a widely used chemical cancer model. In

this approach tumour initiation is achieved with a single dose of the carcinogen DMBA, which leads to somatic *H-Ras* mutation and oncogene activation in a few selected keratinocytes. These cells undergo clonal expansion upon repeated applications of the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), which stimulates the production of growth factors and chronic inflammation, leading to proliferation of the mutant cell population, and precipitating spontaneous secondary mutations in other cancer genes such as *p53*. As the DMBA-initiated keratinocytes can persist in the skin for a long time without forming tumours unless TPA is given, they are thought to represent the epidermal stem cell population, or 'transit amplifying cells' that can divide a small number of times before undergoing terminal differentiation (Owens, Wei, & Smart, 1999; Owens & Watt, 2003; Perez-Losada, & Balmain, 2003) The chemical treatments lead to epidermal hyperplasia and the outgrowth of highly differentiated benign papillomas. Approximately 10% of these papillomas progress to malignant squamous cell carcinomas (SCC), which can disseminate into the sentinel lymph nodes via the lymphatic vessels and further to distant organs via the lymphatic and blood vessels. A small portion of the SCCs can go through an epithelial to mesenchymal transition to the most aggressive and invasive phenotype, spindle cell carcinoma (Yuspa, 1991).

There is significant variability in papilloma formation between mouse strains in the DMBA-TPA model. The outbred SENCAR strain, for example, is highly sensitive to the initiation-promotion regimen, whereas the inbred FVB/N strain shows intermediate sensitivity and C57BL/6 mice are completely resistant to this protocol. On the other hand, both FVB/N and C57BL/6 mice are susceptible to a complete carcinogenesis protocol with repeated DMBA applications (Hennings et al., 1993). The gender disparity often seen in human cancers, and also in experimental cancer models, applies to the DMBA-TPA model as well, as male mice show a higher tumour incidence than females (Balbin et al., 2003; personal observation).

The DMBA-TPA protocol is one of the oldest experimental cancer models. It has provided the data from which many basic concepts of carcinogenesis have been formulated, and despite the long duration and labour-extensiveness of the experiments, still continues to play an important role in understanding the multistage nature of cancer and the mechanisms of inflammation-associated tumour growth (Loeb & Harris, 2008). By subjecting mice modified genetically for collagen XVIII and endostatin to a DMBA-TPA regimen we have found that, besides inhibiting angiogenesis and primary tumour growth, collagen XVIII/endostatin can regulate tumour initiation, tumour-associated inflammation, lymphangiogenesis and lymph node metastasis (Brideau et al., 2007 and unpublished data).

5.6 Genetic Models

The development of advanced molecular technologies that allow manipulation of the mouse genome has provided enormous opportunities for investigating the functions of specific genes in cancer and to create mouse models that mimic human cancers. Genetic tumour models are now available for many common human cancers,

some of which faithfully simulate the human disease (see http://emice.nci.nih.gov or http://tumor.informatics.jax.org/mtbwi/index.do). These can be classified simply as transgenic models in which oncogene or dominant-negative tumour suppressor gene expression is driven by ectopic promoter and enhancer elements and models with targeted gene alterations generated by homologous recombination in which tumour suppressor genes are inactivated or selected mutations are introduced in cancer genes or their modifier genes (reviewed by Hahn & Weinberg, 2002; Herzig & Christofori, 2002; Rangarajan & Weinberg, 2003; Maddison & Clarke, 2005; Frese & Tuveson, 2007).

5.6.1 Transgenic Models

The first transgenic tumour models were established in the mid-1980's by overexpression of single viral or cellular oncogenes under tissue-specific promoters (Macleod & Jacks, 1999; Becher & Holland, 2006). Classical examples of these are the models for mammary tumours and B-cell lymphomas in which c-Myc was expressed under the control of the murine mammary tumour virus (MMTV) promoter and immunoglobulin enhancers, respectively (Stewart, Pattengale, & Leder, 1984; Adams et al., 1985). The most relevant of these early transgenic models, such as the RIP-Tag mouse pancreatic tumour model in which the SV40 large T antigen is expressed under the rat insulin promoter (Hanahan, 1985) are still widely used and have also been appropriately revised to allow more precise evaluation of the stepwise progression of tumour development (Du, Lewis, Hanahan, & Varmus, 2007). These rather simple transgenic models have given valuable information on many principles of tumorigenesis, and, importantly, prepared the ground for more sophisticated genetic models.

Universal problems associated with all traditional transgenic mouse models are the random nature of transgene integration and the use of minimal regulatory elements to drive transgene expression, both of which may result in inaccurate regulation of the target gene. This may cause incomplete penetrance of the desired trait, for example, and/or variability in transgene expression levels. A problem often confronted in cancer research is constitutive expression of the transgene with a potential for affecting other cellular processes and mouse development. This disadvantage can be overcome by means of inducible promoters that allow cancer gene expression to be controlled spatially and temporally, and currently also reversibly. The most widely used system for generating mutant mice with inducible transgene expression employs the tetracycline and doxycycline-controlled transcriptional regulatory elements of *E. coli* (Zhu, Zheng, Lee, Homer & Elias, 2002), while another way is to fuse the oncogene with the oestrogen receptor (ER), making its expression regulatable via oestrogen or tamoxifen ligands (Frese & Tuveson, 2007). These models have been used to demonstrate the necessity for oncogene expression in tumour maintenance, for example (Maddison & Clarke, 2005; Frese & Tuveson, 2007). Suitable transgenic models for our topic of interest, SCC of the skin, include the K14-HPV16 model, in which the early region of human papillomavirus type

16 is targeted to the basal cells of the squamous epithelium (Arbeit, Munger, Howley, & Hanahan, 1994), and the inducible Involucrin-c-myc^{ER} model, targeting the suprabasal epidermal keratinocytes (Pelengaris, Littlewood, Khan, Elia, & Evan, 1999).

5.6.2 Models with Targeted Gene Alterations

Targeted inactivation of tumour suppressor genes, including *p53* and *Rb*, by homologous recombination in embryonic stem cells was achieved in the early 1990's (Donehower et al., 1992; Jacks et al., 1992). Loss of these tumour suppressors in the mouse often resulted in a rather different tumour spectrum from that observed in humans, however. For example, mutations in the human *RB1* gene cause retinoblastoma in childhood followed by osteosarcomas and small cell lung cancer at an adult age, but inactivation of the *Rb* locus in mice led to embryonic lethality, and heterozygous mice developed pituitary and thyroid tumours. Retinal tumours were nevertheless observed in mutant mice when both *Rb* and the closely related *p107* gene were disrupted (Classon & Harlow, 2002).

Similarly, germline inactivation of *p53* in the mouse resulted in an increased risk of developing mainly lymphomas and soft-tissue sarcomas, whereas the spectrum of human tumours with *TP53* mutations also includes breast, brain, colon and lung tumours (Jacks et al., 1994; Hahn & Weinberg, 2002). Numerous genetic tumour models involving ubiquitous or tissue-specific transgenic overexpression of p53 in a dominant-negative manner, and also total or conditional knock-out or knock-in models in which common human p53 mutations have been introduced into the mouse genome, have subsequently shown that the dosage of p53 protein and the type of mutation have profound effects on disease phenotypes. Moreover, p53 knock-out crosses with other tumour-prone strains either overexpressing oncogenes (c-Myc, Wnt-1, Ras) or lacking tumour suppressors (Rb, Nf1, Apc, Brca-1/2) markedly affect tumour susceptibility and the tumour spectrum (Attardi & Donehower, 2005). As mentioned above, telomerase-deficient mice that are heterozygous for *p53* develop breast, colon and skin carcinomas upon ageing instead of the lymphomas and sarcomas which are common in the absence of the *p53* allele alone (Artandi et al., 2000). These mice with aberrant expression of tumour suppressors p53 and Rb, represent just a few examples of classical targeted tumour models. An ample repertoire of genetic models involving changes in the critical cellular processes in tumour development (including self-sufficiency/proliferation, apoptosis/survival, genomic instability, senescence, angio/lymphangiogenesis, invasion and tissue remodelling) and a number of cancer genes have been generated during the last 20 years, some of which have resulted in a very different tumour spectrum from that expected (Hahn & Weinberg, 2002; Herzig & Christofori, 2002; Maddison & Clarke, 2005; Frese & Tuveson, 2007; see also http://emice.nci.nih.gov and http://tumor.informatics.jax.org/mtbwi/index.do).

There are certain severe limitations associated with basic models with targeted gene alterations (and obviously also with transgenic models) in which a single

genetic change is introduced into the mouse genome in order to mimic human cancer. As discussed above, tumour development requires multiple stepwise genetic and epigenetic changes in epithelial cells, which cannot be achieved in constitutive knock-out models. In addition, most human tumours result from sporadic mutations in proto-oncogenes or tumour suppressor genes in somatic cells, whereas in traditional mouse models the modifications are introduced into the germline and may thus influence mouse development as well (Herzig & Christofori, 2002; Maddison & Clarke, 2005).

Conditional mutagenesis, which allows spatiotemporal mutations to be introduced into the mouse genome, has partially resolved these shortcomings. Conditional mutants are generated by means of Cre-Lox or yeast-derived FLP-FRT systems, in which bacterial Cre or yeast-derived FLT recombinase, driven by a tissue-specific promoter, catalyses a recombination event that results in the desired mutation in selected cells at a controlled point in time. These systems can be further fine-tuned by controlling Cre expression through ligand-induced systems such as CreER, or by delivering Cre into the tissue using adenoviral vectors, for example (Macleod & Jacks, 1999; Maddison & Clarke, 2005; Frese & Tuveson, 2007). Gut-specific homozygous inactivation of the *Apc* tumour suppressor gene which circumvents the embryonic lethality of total Apc deficiency represents a pioneer work aimed at producing a conditional model for tumour development (Shibata et al., 1997). Other improvements in genetic models include the generation of a mouse strain in which *K-ras* oncogene activation is achieved in just a few cells by means of spontaneous recombination (the "hit and run" strategy), mimicking the sporadic formation of human cancers (Johnson et al., 2001). Restricted somatic mutations can also be achieved by delivering oncogenes by retroviral infection to transgenic mice engineered to express a receptor for avian retrovirus (tumor virus A, TVA) under cell-type-specific promoters. In this way multiple genetic changes can be sequentially introduced into somatic cells by multiple rounds of retrovirus infection, thereby mimicking more accurately the multistage nature of human cancers and the consecutive changes involved. This approach has been used to study the cooperation between activated K-ras and Akt oncogens in glioblastomas (Holland, 2000). Furthermore, the retrovirus strategy has been successfully combined with traditional genetic tumour models such as RIP-Tag to allow detailed studies on multistep cancer progression (Du, Lewis, Hanahan & Varmus, 2007).

5.7 Concluding Remarks

Experimental tumour modelling in mice has been extended from simple chemical carcinogenesis models and transplanted tumours to complicated genetic models generated by advanced molecular technologies and with a potential for more accurate recapitulation of the changes that occur in human cancers. Some of the models can also be used to investigate tumour metastasis, while others are more suitable for monitoring primary tumour growth only, an issue discussed in detail elsewhere (see Khanna & Hunter, 2005; Kim & Baek, 2010). Common or novel mutations that are

found in human tumours, or their combinations, can be introduced into the mouse genome to validate their contribution to tumour formation and progression in vivo. These models can be used to explore the detailed molecular mechanisms underlying tumour development, and also to an increasing extent for targeted drug testing. Nevertheless, even if the novel genetic models appear to provide better opportunities to study different aspects of tumorigenesis, the traditional mouse tumour models are most likely to maintain their position in many applications, particularly for large-scale drug testing. In addition, some of the old models have been reintroduced and combined with transgenic and knock-out models, and have proved very useful in intricate and challenging cancer studies. Examples of these include the classical DMPA-TPA protocol for mouse skin carcinogenesis applied to mice with mutations for cancer genes to explore the contribution of these to tumour-associated inflammation and skin cancer of stem cell origin. As discussed thoroughly above, all experimental models have their strengths and weaknesses, and eventually it is the biological or translational problem that dictates which model or models should be employed to obtain reliable answers to that particular question.

References

Adams, J. M., Harris, A. W., Pinkert, C. A., Corcoran, L. M., Alexander, W. S., Cory, S., et al. (1985). The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature*, *318*, 533–538.

Alvarez, E. (2002). B16 murine melanoma: Historical perspective on the development of a solid tumor model. In B. A. Teicher (Ed.), *Tumor models in cancer research* (pp. 73–89). Totowa, NJ: Humana Press.

Anisimov, V. N., Ukraintseva, S. V., & Yashin, A. I. (2005). Cancer in rodents: Does it tell us about cancer in humans? *Nature Reviews Cancer*, *5*, 807–819.

Arbeit, J. M., Munger, K., Howley, P. M., & Hanahan, D. (1994). Progressive squamous cell epithelial neoplasia in K14-human papillomavirus type 16 transgenic mice. *Journal of Virology*, *68*, 4358–4368.

Artandi, S. E., Chang, S., Lee, S. L., Alson, S., Gottlieb, G. J., Chin, L., et al. (2000). Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature*, *406*, 641–645.

Attardi, L. D., & Donehower, L. A. (2005). Probing p53 biological functions through the use of genetically engineered mouse models. *Mutation Research*, *576*, 4–21.

Balbin, M., Fueyo, A., Tester, A. M., Pendas, A. M., Pitiot, A. S., Astudillo, A., et al. (2003). Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. *Nature Genetics*, *35*, 252–257.

Becher, O. J., & Holland, E. C. (2006). Genetically engineered models have advantages over xenografts for preclinical studies. *Cancer Research*, *66*(7), 3355–3358.

Berger, M. R. (1999). Autochthonous tumour models in rats: Is there a relevance for anticancer drug development. In H. H. Fiebig & A. M. Burger (Eds.), *Relevance of tumour models for anticancer drug development* (pp. 15–27). Contrib Oncol 54. Basel: Karger.

Bibby, M. C. (2004). Orthotopic models of cancer for preclinical drug evaluation: Advantages and disadvantages. *The European Journal of Cancer*, *40*, 852–857.

Brideau, G., Makinen, M. J., Elamaa, H., Tu, H., Nilsson, G., Alitalo, K., et al. (2007). Endostatin overexpression inhibits lymphangiogenesis and lymph node metastasis in mice. *Cancer Research*, *67*, 11528–11535.

Classon, M., & Harlow, E. (2002). The retinoblastoma tumour suppressor in development and cancer. *Nature Reviews Cancer*, *2*, 910–917.

Corbett, H. T. (2002). Transplantable syngeneic rodent tumors: Solid tumors in mice. In B. A. Teicher (Ed.), *Tumor models in cancer research* (pp. 23–40). Totowa, NJ: Humana Press.

DePinho, R. A. (2000). The age of cancer. *Nature*, *408*, 248–254.

Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr, Butel, J. S., et al. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, *356*, 215–221.

Du, Y. C., Lewis, B. C., Hanahan, D., & Varmus, H. (2007). Assessing tumor progression factors by somatic gene transfer into a mouse model: Bcl-xL promotes islet tumor cell invasion. *PLoS Biology*, *5*, e276.

Folkman, J. (2006). Antiangiogenesis in cancer therapy-endostatin and its mechanisms of action. *Experimental Cell Research*, *312*, 594–607.

Frese, K. K., & Tuveson, D. A. (2007). Maximizing mouse cancer models. *Nature Reviews Cancer*, *7*, 645–658.

Giovanella, B. C., Yim, S. O., Stehlin, J. S., & Williams, L. J., Jr (1972). Development of invasive tumors in the "nude" mouse after injection of cultured human melanoma cells. *Journal of the National Cancer Institute*, *48*, 1531–1533.

Hahn, W. C., & Weinberg, R. A. (2002). Modelling the molecular circuitry of cancer. *Nature Reviews Cancer*, *2*, 331–341.

Hanahan, D. (1985). Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature*, *315*, 115–122.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, *100*, 57–70.

Hennings, H., Glick, A. B., Lowry, D. T., Krsmanovic, L. S., Sly, L. M., & Yuspa, S. H. (1993). FVB/N mice: An inbred strain sensitive to the chemical induction of squamous cell carcinomas in the skin. *Carcinogenesis*, *14*, 2353–2358.

Herzig, M., & Christofori, G. (2002). Recent advances in cancer research: Mouse models of tumorigenesis. *Biochimica et Biophysica Acta*, *1602*, 97–113.

Hoffman, R. M. (1999). Orthotopic metastatic mouse models for anticancer drug discovery and evaluation: A bridge to the clinic. *Investigational New Drugs*, *17*, 343–359.

Hoffman, R. M. (2005). The multiple uses of fluorescent proteins to visualize cancer in vivo. *Nature Reviews Cancer*, *5*, 796–806.

Holland, E. C. (2000). A mouse model for glioma: Biology, pathology, and therapeutic opportunities. *Toxicologic Pathology*, *28*, 171–177.

Jacks, T., Fazeli, A., Schmitt, E. M., Bronson, R. T., Goodell, M. A., & Weinberg, R. A. (1992). Effects of an Rb mutation in the mouse. *Nature*, *359*, 295–300.

Jacks, T., Remington, L., Williams, B. O., Schmitt, E. M., Halachmi, S., Bronson, R. T., et al. (1994). Tumor spectrum analysis in p53-mutant mice. *Current Biology*, *4*, 1–7.

Jacob, D. (2004). Xenograftic tumor models in mice for cancer research, a technical review. *Gene Therapy and Molecular Biology*, *8*, 213–219.

Johnson, L., Mercer, K., Greenbaum, D., Bronson, R. T., Crowley, D., Tuveson, D. A., et al. (2001). Somatic activation of the K-ras oncogene causes early onset lung cancer in mice. *Nature*, *410*, 1111–1116.

Khanna, C., & Hunter, K. (2005). Modeling metastasis in vivo. *Carcinogenesis*, *26*, 513–523.

Kim, I. S., & Baek, S. H. (2010). Mouse models for breast cancer metastasis. *Biochemical and Biophysical Research Communications*, *394*, 443–447.

Kulke, M. H., Bergsland, E. K., Ryan, D. P., Enzinger, P. C., Lynch, T. J., Zhu, A. X., et al. (2006). Phase II study of recombinant human endostatin in patients with advanced neuroendocrine tumors. *Journal of Clinical Oncology*, *24*, 3555–3561.

Loeb, L. A., & Harris, C. C. (2008). Advances in chemical carcinogenesis: A historical review and prospective. *Cancer Research*, *68*, 6863–6872.

Luch, A. (2005). Nature and nurture – Lessons from chemical carcinogenesis. *Nature Reviews Cancer*, *5*, 113–125.

Macleod, K. F., & Jacks, T. (1999). Insights into cancer from transgenic mouse models. *The Journal of Pathology*, *187*, 43–60.

Maddison, K., & Clarke, A. R. (2005). New approaches for modelling cancer mechanisms in the mouse. *The Journal of Pathology*, *205*, 181–193.

Mahler, J. F., Stokes, W., Mann, P. C., Takaoka, M., & Maronpot, R. R. (1996). Spontaneous lesions in aging FVB/N mice. *Toxicologic Pathology*, *24*, 710–716.

Manzotti, C., Audisio, R. A., & Pratesi, G. (1993). Importance of orthotopic implantation for human tumors as model systems: Relevance to metastasis and invasion. *Clinical and Experimental Metastasis*, *11*, 5–14.

Moschos, S. J., Odoux, C., Land, S. R., Agarwala, S., Friedland, D., Volker, K. M., et al. (2007). Endostatin plus interferon-alpha2b therapy for metastatic melanoma: A novel combination of antiangiogenic and immunomodulatory agents. *Melanoma Research*, *17*, 193–200.

Moser, A. R., Pitot, H. C., & Dove, W. F. (1990). A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science*, *247*, 322–324.

Owens, D. M., & Watt, F. M. (2003). Contribution of stem cells and differentiated cells to epidermal tumours. *Nature Reviews Cancer*, *3*, 444–451.

Owens, D. M., Wei, S., & Smart, R. C. (1999). A multihit, multistage model of chemical carcinogenesis. *Carcinogenesis*, *20*, 1837–1844.

Paigen, K. (2003). One hundred years of mouse genetics: An intellectual history. II. The molecular revolution (1981–2002). *Genetics*, *163*, 1227–1235.

Pelengaris, S., Littlewood, T., Khan, M., Elia, G., & Evan, G. (1999). Reversible activation of c-Myc in skin: Induction of a complex neoplastic phenotype by a single oncogenic lesion. *Molecular Cell*, *3*, 565–577.

Perez-Losada, J., & Balmain, A. (2003). Stem-cell hierarchy in skin cancer. *Nature Reviews Cancer*, *3*, 434–443.

Peters, L. L., Robledo, R. F., Bult, C. J., Churchill, G. A., Paigen, B. J., & Svenson, K. L. (2007). The mouse as a model for human biology: A resource guide for complex trait analysis. *Nature Reviews Genetics*, *8*, 58–69.

Rangarajan, A., & Weinberg, R. A. (2003). Opinion: Comparative biology of mouse versus human cells: Modelling human cancer in mice. *Natures Reviews Cancer*, *3*, 952–959.

Russel, W. M. S., & Burch, R. L. (1992). *The principles of humane experimental technique*. Potters Bar: Universities Federation For Animal Welfare.

Schuh, J. C. (2004). Trials, tribulations, and trends in tumor modeling in mice. *Toxicologic Pathology* , *32*, 53–66.

Sharma, S., Kelly, T. K., & Jones, P. A. (2010). Epigenetics in cancer. *Carcinogenesis*, *31*, 27–36.

Shibata, H., Toyama, K., Shioya, H., Ito, M., Hirota, M., Hasegawa, S., et al. (1997). Rapid colorectal adenoma formation initiated by conditional targeting of the *Apc* gene. *Science*, *278*, 120–123.

Shultz, L. D., Ishikawa, F., & Greiner, D. L. (2007). Humanized mice in translational biomedical research. *Nature Reviews Immunology*, *7*, 118–130.

Stewart, T. A., Pattengale, P. K., & Leder, P. (1984). Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. *Cell*, *38*, 627–637.

Suggitt, M., & Bibby, M. C. (2005). 50 years of preclinical anticancer drug screening: Empirical to target-driven approaches. *Clinical Cancer Research*, *11*, 971–981.

Sund, M., Hamano, Y., Sugimoto, H., Sudhakar, A., Soubasakos, M., Yerramalla, U., et al. (2005). Function of endogenous inhibitors of angiogenesis as endothelium-specific tumor suppressors. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 2934–2939.

Talmadge, J. E., Singh, R. K., Fidler, I. J., & Raz, A. (2007). Murine models to evaluate novel and conventional therapeutic strategies for cancer. *The American Journal of Pathology*, *170*, 93–804.

Yuspa, S. H. (1991). Cutaneous carcinogenesis: Natural and experimental. In L. A. Goldsmith (Ed.), *Physiology, biochemistry and molecular biology of the skin* (pp. 1365–1402). Oxford: Oxford University Press.

Zhu, Z., Zheng, T., Lee, C. G., Homer, R. J., & Elias, J. A. (2002). Tetracycline-controlled transcriptional regulation systems: Advances and application in transgenic animal modeling. *Seminars in Cell & Developmental Biology*, *13*, 121–128.

Chapter 6
Exploration of MMP Function in Mouse Models of Angiogenesis

Anne Masset, Mehdi El Hour, Sarah Berndt, Nor Eddine Sounni, and Agnès Noel

Abstract Angiogenesis research has become one of the most important areas in the biomedical field. The fast expansion of this sphere of research requires the development of reliable and reproductive models applicable to the increasing number of knock-in or knock-out mice that are now available. Here, we discuss several commonly used *ex vivo* and in vivo models by illustrating how they pointed out the functions of matrix metalloproteinases (MMPs) during the complex angiogenic process associated with cancer progression and dissemination.

6.1 Introduction

The onset of tumor formation is based on a combination of genetic and epigenetic alterations that activate oncogenes and/or inactivate tumor suppressor genes. These alterations lead to increased proliferation rate and decreased apoptosis in cancer cells resulting in local cancer expansion. For many years, neoplastic cells have been the focus of interest in cancer research. However, this tumor cell-centered view of cancer development has largely ignored the contribution of the tumor microenvironment to the malignant phenotype. It is now clear that as important as tumor cells are the cellular and molecular components of the environment that tumor cells encounter as they progress throughout the body. Host cells infiltrating the tumor include blood or lymphatic endothelial cells, pericytes, smooth muscle cells (myo)fibroblasts, adipocytes, immune and inflammatory cells (Fig. 6.1) (Albini & Sporn, 2007; Mueller & Fusenig, 2004; Noel, Jost & Maquoi, 2008; Tlsty, 2001). The non-cellular tumor compartment consists of the various components of the extracellular matrix (ECM), whose composition directly and indirectly influences the phenotype of tumor cells and host cells.

A. Noel (✉)
Groupe Interdisciplinaire de Génoprotéomique Appliquée-Cancer (GIGA-Cancer), Laboratory of Tumor and Developmental Biology, University of Liege, B-4000 Liège, Belgium
e-mail: agnes.noel@ulg.ac.be

Anne Masset, and Mehdi El Hour are Equally contributed

C. Brakebusch, T. Pihlajaniemi (eds.), *Mouse as a Model Organism*,
DOI 10.1007/978-94-007-0750-4_6,

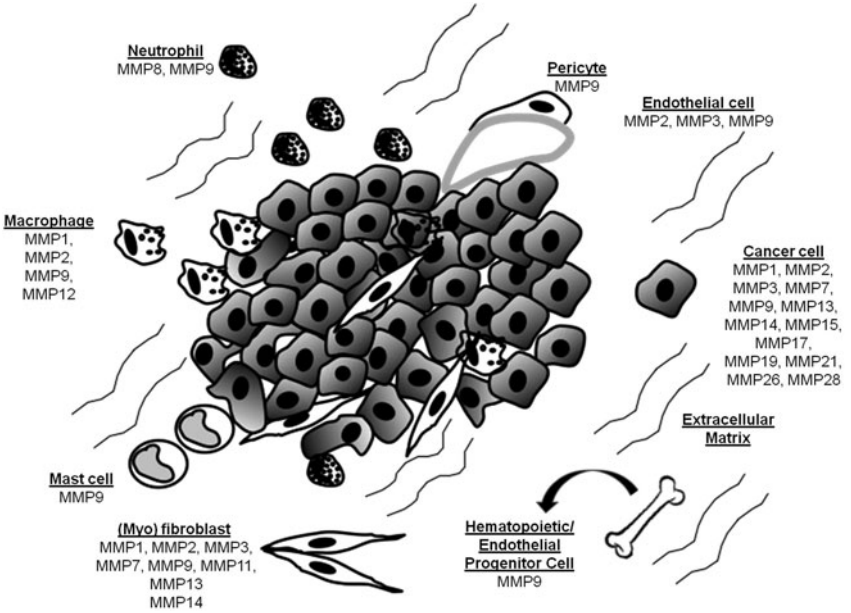

Fig. 6.1 MMP production in primary tumor. Primary tumor is composed of different cell types including cancer cells, inflammatory cells, endothelial cells and fibroblasts. All these cells secrete soluble MMPs or produce membrane anchored MMPs (MMP14-17, MM24, MMP25). Some cells can be recruited from bone marrow as progenitor cells (hematopoietic and/or endothelial progenitor cells). MMPs can either promote tumor progression or abolish it

Once the tumoral mass has reached a critical volume, tumor cells located at distance from blood vessels are not anymore supplied by nutrients and oxygen and are susceptible to apoptosis. However, some tumor cells acquire the ability to overcome this growth inhibition by activating specific hypoxic-signaling pathways and inducing angiogenesis (Otrock, Hatoum, Awada, Ishak & Shamseddine, 2009), the formation of new blood vessels. Such a transition from an avascular (pre-vascular) phase characterizing a dormant tumor to a vascular phase is referred to as the "angiogenic switch" (Baeriswyl & Christofori, 2009; Bergers & Benjamin, 2003; Ribatti, Nico, Crivellato, Roccaro & Vacca, 2007). This is a time-restricted event where the balance between pro- and anti-angiogenic factors (Baeriswyl & Christofori, 2009) tilts in favour of pro-angiogenic molecules. The "angiogenic switch" is a key determinant of the multistage tumor progression promoting not only tumor growth, but also tumor cell dissemination through blood vessels. This determinant step of the cancer development and dissemination involves important tissue remodellings implicating various proteases like serine proteases (plasmin/plasminogen activator), cysteine proteases (B-cathepsin, L-cathepsin), aspartic proteases (D-cathepsin) and metalloproteinases (MMPs, ADAMs for a disintegrin and metalloproteinase and ADAMTSs for a disintegrin and metalloproteinase with thrombospondin motifs) (Egeblad & Werb, 2002; Lopez-Otin & Overall, 2002; Overall, 2004). Although

initially viewed as simple regulators of matrix destruction, these proteases are now recognized as active players during the different steps of cancer growth and invasion (Page-McCaw, Ewald & Werb, 2007; Roy, Yang & Moses, 2009). A special emphasis has been recently given on MMPs which emerged as positive or even negative regulators of angiogenesis (Lopez-Otin & Matrisian, 2007; Martin & Matrisian, 2007; Noel et al., 2008). MMPs were thought to function mainly as enzymes that degrade structural components of the ECM. However, beyond the ECM molecules, there is a growing list of MMP substrates that includes growth factors, tyrosine kinase receptors, other MMPs, cytokines and chemokines, as well as cell-cell and cell-matrix adhesion molecules (Page-McCaw et al., 2007). Most MMPs are secreted as soluble enzyme, but several are membrane type MMPs (MT-MMPs) which are associated with the cell membrane by either a carboxy-terminal transmembrane domain (MT1-MMP or MMP-14, MT2-MMP or MMP-15, MT3-MMP or MMP-16, MT5-MMP or MMP24) or a glycosylphosphatidyl-inositol (GPI) anchor (MT4-MMP or MMP17 and MT6-MMP or MMP25) (Fanjul-Fernandez, Folgueras, Cabrera & Lopez-Otin, 2009; Gomis-Ruth, 2009). They can be produced both by tumor cells and host cells (Noel et al., 2008) (Fig. 6.1).

In this review, we present the most relevant in vivo models applicable to mice that have proven usefulness in exploring the angiogenic process. We illustrate the interest of these models by describing the new insights into MMP functions given by these models. For a general description of MMPs, the reader is referred to previous reviews (Chabottaux & Noel, 2007; Fanjul-Fernandez et al., 2009; Mott & Werb, 2004; Roy et al., 2009). General descriptions of in vitro models of angiogenesis are available (Bruyere & Noel, 2010; Jensen, Cao & Cao, 2009; Berndt, Bruyère, Jost, Edwards & Noël, 2008; Jost et al., 2008). The *ex vivo* and in vivo models used for MMP investigation in mice and presented here include: aortic ring assay, Matrigel Plug assay and the transplantation chamber assay (Fig. 6.2). We also discuss three transgenic mouse model of spontaneous carcinogenesis employed as reliable tools to study the angiogenic switch: Rip1-Tag2, keratin-14 (K14) HPV16 (human papilloma virus) and MMTV PyMY mice.

6.2 The Aortic Ring Assay

The aortic ring model has become one of the most widely used systems to study angiogenesis and its mechanisms (Aplin, Fogel, Zorzi & Nicosia, 2008; Nicosia, 2009). This system consists of culturing fragments of mice aorta into a type I collagen gel. Endothelial cells that originated mostly from the two wounded edges of the aortic rings are organized into capillaries which can be covered by pericytes and/or smooth muscle cells (Berndt et al., 2006; Berndt et al., 2008). This model has gained broad acceptance since it bridges the gap between in vitro and in vivo models (Berndt et al., 2008; Nicosia, 2009). This assay recapitulates different events of sprouting angiogenesis and takes into account the multicellular composition of the vessel wall (Fig. 6.2). In this model, MMP dependence has been assessed by the blockade of the angiogenic response by adding synthetic MMP inhibitors (BB94 or

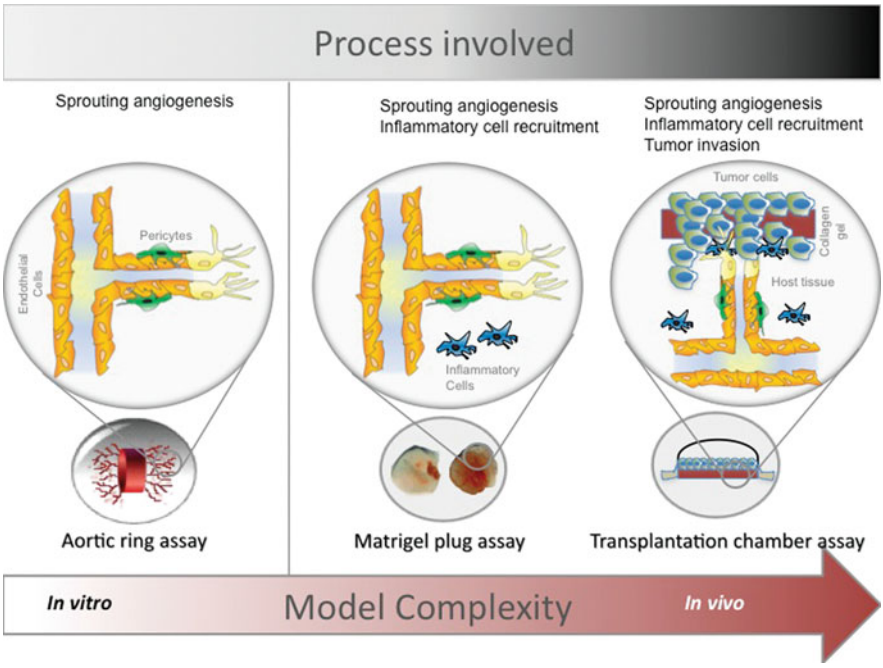

Fig. 6.2 Models complexity from in vitro to in vivo assays of angiogenesis. Schematic representation of in vitro and in vivo models used to study the different roles of MMPs during tumoral angiogenesis. Each model depicting the cellular actors engaged is ranked on the basis of its complexity of cellular interactions

batimastat, BB-2516 or marimastat, GM6001 or ilomastat (Galardin), RO-28-2653) at the beginning of the experiment (Burbridge et al., 2002; Chun et al., 2004; Maquoi et al., 2004; Zhu, Guo, Villaschi & Francesco Nicosia, 2000). In addition, Batimastat and Marimastat stabilized the microvessels and prevented vascular regression after the angiogenic growth phase (Burbridge et al., 2002; Zhu et al., 2000).

The individual function of different MMPs has been investigated by applying the model to different MMP-deficient mice. MMP-11 and MMP-19 are not required for endothelial cell spreading out from the aortic rings. Similarly, a single or a combined lack of MMP-2 and MMP-9 does not impair the in vitro capillary outgrowth from aortic rings (Aplin, Zhu, Fogel & Nicosia, 2009; Chun et al., 2004; Masson et al., 2005). However, MMP-2 deficiency inactivates the VEGF-induced angiogenic response (Cheng et al., 2007). Among different MMPs investigated in the aortic ring assay, MT1-MMP appears as a key regulator of the angiogenic sprouting since its gene deficiency leads to defective angiogenic response (Aplin et al., 2009; Chun et al., 2004). Weiss and co-workers demonstrated that MT1-MMP focus lytic activity to the invadopodia of migrating cells and allows cellular invasion in collagen matrix (Yana et al., 2007). MT1-MMP can also contribute to the angiogenic process

in cancer through the processing of cell surface molecules and the enhancement of VEGF gene expression by tumor cells (Sounni et al., 2004).

6.3 The Matrigel Plug Assay

In this model, matrigel supplemented with either cells or angiogenic factors is injected sub-cutaneously into mice and the plug is removed after 7–21 days (Berndt et al., 2008). Plug vascularization can be quantified either by histochemical analysis or by measurement of their hemoglobin contents (Fig. 6.2). In this system, the oral administration of a broad spectrum MMP inhibitor (BAY12-9566) inhibits FGF-induced angiogenesis (Berndt et al., 2006; Wojtowicz-Praga, 1999). The injection of MMP-9 antisense in mice also decreases plug vascularization (London et al., 2003). Surprisingly, the angiogenic response is increased rather than decreased in MMP $19^{-/-}$ mice (Jost et al., 2006). This observation further supports the opposite effects of various MMPs during the process of angiogenesis, some being pro-angiogenic agents (MMP-9) and other acting as negative regulators (MMP-19) of angiogenesis (Lopez-Otin & Matrisian, 2007).

6.4 The Transplantation Chamber Assay

The transplantation chamber assay allows the reconstitution of a skin carcinoma under the influence of the connective tissue environment. Mouse malignant keratinocytes are pre-cultured on a type I collagen gel mounted between two concentric Teflon rings. The culture is then covered by a silicone transplantation chamber and transplanted in toto onto the back muscle fascia of mice (Fig. 6.2). The collagen gel is gradually infiltrated by host cells and replaced by a highly vascularized granulation tissue (Mueller & Fusenig, 2004). One important finding of studies using this transplantation system is that the invasive growth, the hallmark of malignancy, does not manifest until the vascularized host tissue has replaced the gel and get into contact with the tumor cells (Mueller & Fusenig, 2004; Skobe, Rockwell, Goldstein, Vosseler & Fusenig, 1997).

The single deficiency of MMP-2, MMP-3, MMP-8, MMP-9 or MMP-11 does not affect tumor invasion and angiogenesis in this model (Masson et al., 2005). In sharp contrast, both tumor invasion and vascularization are impaired by the combined deficiency of MMP-2 and MMP-9 (Masson et al., 2005). Of particular importance is the necessity of specific interactions occurring between tumor cells and mesenchymal cells producing MMP-2, as well as inflammatory cells secreting MMP-9 (Masson et al., 2005). Using MMP-$13^{-/-}$ mice, Lederle et al demonstrated an essential role of this collagenase in the invasive growth of skin squamous cell carcinoma (Lederle et al., 2009). MMP-13 produced by stromal fibroblastic cells plays a crucial role in the maintenance of angiogenesis at the invasive stage.

Surprisingly, the angiogenic response was accelerated and tumor invasion increased in MMP-19 knock-out mice (Jost et al., 2006). These findings further support the recent discovery of some MMPs as protective molecules towards cancer progression (Lopez-Otin &Matrisian, 2007).

6.5 Transgenic Mice Models of the Angiogenic Switch

Transgenic mice recapitulate the ontogeny of tumorigenesis, often through a series of reproducible temporal and histological stages that are accessible to molecular analysis. One advantage of such mice models ("onco" mice) is to offer the opportunity to evaluate the impact of a genetic ablation during the whole process of cancer progression and dissemination, while it is temporally assessed in other models, such as tumor cell xenografts. However, transgenic mice are time-consuming, expensive, strain background-dependent and the implication of inflammatory reactions in these systems renders complex the significance interpretation of the cellular and molecular mechanisms. In addition, genetic ablation is present from the first step of development and compensatory increase of (an) other modulator(s) could take place during mouse development and growth. Despite these limitations, crossing "onco" mice with MMP-deficient mice have provided new insights into the cellular source of MMPs and the multifunctional features of MMPs. In this review, we present three transgenic mouse models that have been instrumental in providing the proof of concept of the angiogenic switch (Baeriswyl & Christofori, 2009; Ribatti et al., 2007).

(a) K14-HPV16 Mouse Model of Skin Carcinoma

K14-HPV16 transgenic mice express early region genes (E6 and E7) of the human papillomavirus type 16 in basal keratinocytes under the control of the keratin 14 promoter/enhancer (Arbeit, Munger, Howley & Hanahan, 1994; Coussens, Hanahan & Arbeit, 1996). K14-HPV16 mice show multistage development of invasive squamous cell carcinoma (SCC) of the epidermis. By 1 month of age, K14-HPV16 mice develop epidermal hyperplasia with 100% penetrance characterized by a terminally differentiating hyperproliferative epidermis. Hyperplastic lesions advance focally into angiogenic dysplasia between 3 and 6 months of age and are distinct from hyperplasia based upon prominent hyperproliferative epidermis that fails to undergo terminal differentiation. After 8–12 months of age, dysplasia undergoes malignant conversion into different grades of skin squamous cell carcinoma in 50% of mice. Among them, 30% metastasize to regional lymph nodes (Arbeit et al., 1994; Coussens et al., 1996). This model has provided crucial information on the implication of MMP-9 and a tissue inhibitor of metalloproteinases (TIMP-1) on tumoral progression. TIMP-1 enhances tumorigenicity by potentiating keratinocyte hyperproliferation and accelerated the appearance of genomic copy number abnormalities

in early premalignant cells, thereby enhancing their susceptibility to undergo malignant conversion (Rhee, Diaz, Korets, Hodgson & Coussens, 2004). Crossing of MMP-9-deficient mice with K14-HPV16 transgenic mice has demonstrated the requirement of MMP-9 activity for epithelial carcinogenesis. HPV16/MMP-$9^{-/-}$ mice are characterized by (1) a delay in the development of hyperplastic and dysplastic phenotypes, (2) a reduction in the incidence of carcinoma, (3) an attenuation of keratinocyte proliferation and (4) a delay on angiogenesis (Coussens, Tinkle, Hanahan & Werb, 2000). This study also demonstrated that inflammatory cells (macrophages, mast cells and neutrophils) are an important class of tumor-associated cells (Tlsty & Coussens, 2006). Bone marrow-derived inflammatory cells are the predominant source of MMP-9. Interestingly, the bone marrow transplantation of MMP-$9^{+/+}$ bone marrow cells into lethally irradiated MMP9-deficient K14-HPV16 mice restores tumor angiogenesis and tumor growth (Coussens et al., 2000).

(b) MMTV-PyMT Mouse Model of Breast Cancer

In MMTV-PyMT mice, tumors are induced by expressing the polyoma middle T oncogene under the mouse mammary tumor virus LTR enhancer (MMTV-PyMT) (Fluck & Schaffhausen, 2009). The PyMT model recapitulates many processes found in human breast cancer progression not only morphogically but also in the pattern of expression of biomarkers associated with poor prognosis (Lin et al., 2003). Mammary tumors occur in 100% PyMT mice as early as 4 weeks, and a large percentage of mice developed carcinoma at 14 weeks. The morphology of PyMT tumors during progression is divided in four-stage classification scheme that includes hyperplasia (100% of penetrance after 4 weeks of age), adenoma/mammary intraepithelial neoplasia (MIN) (7–9 weeks), early carcinoma (between 8 and 12 weeks) and late carcinoma (from 10 weeks) (Bergers & Benjamin, 2003). The tumors spontaneously metastasize to the lungs by ∼90 days with a penetrance of 85%.

Galardin, a potent inhibitor that reacts with most MMPs, significantly reduces primary tumor growth and metastatic dissemination. Primary tumors from galardin-treated mice exhibit a lower histopathological tumor grade, increased collagen deposition and increased MMP2 activity (Almholt et al., 2008). The genetic ablation of MMP-3 does not significantly affect tumor growth and metastasis. MMP-7 expression is not significant in this model whereas MMP-9 produced predominantly by inflammatory cells is necessary for efficient development of metastases (Olson et al., 2009). The contribution of MMP9 is strain-dependent, as FVB/N mice do not show effect of either genetic ablation or pharmacologic inhibition of MMP-9 on lung metastasis, in contrast to C57BL/6 strain mice (Martin et al., 2008). In MT1-MMP-null mammary glands, an increase in primary tumor growth rate is observed, but this was followed by a reduction of lung metastasis (Szabova, Chrysovergis, Yamada & Holmbeck, 2008). The absence of the collagenase MMP-13 does not influence tumor growth, vascularization, or metastasis to the lungs (Nielsen et al., 2008).

(c) Rip1-Tag2 Mouse Model of Pancreatic Cancer

Rip1Tag2 transgenic mice express the large-T antigen (Tag) of simian virus 40 under the control of the rat insulin promoter and elicit tissue-specific expression of the transgene in the endocrine pancreas. T antigen inactivates two important tumor suppressor pathways by interacting with p53 and Rb and inhibiting their functions. These transgenic mice succumb to a well-characterized progression of multistep tumorigenesis occuring specifically in the pancreatic islets of Langerhans. At 4–6 weeks old, a hyperproliferative switch first occurs in 50% of islets resulting in the appearance of hyperplastic/dysplastic islets. By 6–8 weeks, about 25% of these islets are switched on angiogenesis with histological features of high-grade dyplasia. By 12–14 weeks, 100% mice develop highly vascularized tumors that can differ in their invasive capacity (Bergers, Hanahan & Coussens, 1998; Parangi et al., 1996). Inflammatory cells and specially neutrophils are the main source of MMP-9 in RIP1-Tag2 mice and they play a crucial role in activating angiogenesis during the early stages of carcinogenesis (Nozawa, Chiu & Hanahan, 2006). The genetic ablation of MMP-2 and MMP-9 in RIP1-Tag2 mice illustrates an important role of these proteases in tumour growth and angiogenic switch respectively (Bergers et al., 2000).

6.6 Conclusions

The use of mouse models to study human cancer in genetically modified environment has recently unearthed a certain amount of complexity of MMPs in promoting or protecting cancer development. Mechanistic studies and the exploration of individual protease gene functions in various organ-specific mouse models of cancer development are required to depict their unique and/or redundant function. Indeed, there are still a number of organ-specific mouse models of cancer not investigated yet in MMP modified environment such as mouse models of brain, lung, leukemia and prostate cancer (Abate-Shen, 2006). The critical next step will be to determine whether mouse models can be useful to study the cascade of enzymatic activities in a complex molecular network that altogether, foster or abolish neoplastic development. Because of the multiple roles played by MMPs in vivo, further focuses on intersecting proteolytic activities contributing to the overall proteolysis and the cellular and intercellular signalling during carcinogenesis are needed to delineating pathways that can be targeted with anticancer drugs.

References

Abate-Shen, C. (2006). A new generation of mouse models of cancer for translational research. *Clinical Cancer Research*, *12*, 5274–5276.

Albini, A., & Sporn, M. B. (2007). The tumour microenvironment as a target for chemoprevention. *Nature Reviews Cancer*, *7*, 139–147.

Almholt, K., Juncker-Jensen, A., Laerum, O. D., Dano, K., Johnsen, M., Lund, L. R., et al. (2008). Metastasis is strongly reduced by the matrix metalloproteinase inhibitor Galardin in the MMTV-PymT transgenic breast cancer model. *Molecular Cancer Therapeutics*, *7*, 2758–2767.

Aplin, A. C., Fogel, E., Zorzi, P., & Nicosia, R. F. (2008). The aortic ring model of angiogenesis. *Methods in Enzymology*, *443*, 119–136.

Aplin, A. C., Zhu, W. H., Fogel, E., & Nicosia, R. F. (2009). Vascular regression and survival are differentially regulated by MT1-MMP and TIMPs in the aortic ring model of angiogenesis. *American Journal of Physiology – Cell Physiology*, *297*, C471–480.

Arbeit, J. M., Munger, K., Howley, P. M., & Hanahan, D. (1994). Progressive squamous epithelial neoplasia in K14-human papillomavirus type 16 transgenic mice. *Journal of Virology*, *68*, 4358–4368.

Baeriswyl, V., & Christofori, G. (2009). The angiogenic switch in carcinogenesis. *Seminars in Cancer Biology*, *19*, 329–337.

Bergers, G., & Benjamin, L. E. (2003). Tumorigenesis and the angiogenic switch. *Nature Reviews Cancer*, *3*, 401–410.

Bergers, G., Brekken, R., McMahon, G., Vu, T. H., Itoh, T., Tamaki, K., et al. (2000). Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nature Cell Biology*, *2*, 737–744.

Bergers, G., Hanahan, D., & Coussens, L. M. (1998). Angiogenesis and apoptosis are cellular parameters of neoplastic progression in transgenic mouse models of tumorigenesis. *International Journal of Developmental Biology*, *42*, 995–1002.

Berndt, S., Bruyère, F., Jost, M., Edwards, D. R., & Noël, A. (2008). Chapter 16: In vitro and in vivo models of angiogenesis to dissect MMP functions. In D. R. Edwards, G. Hoyer-Hansen, F. Blasi, & B. F. Sloane (Eds), *The cancer degradome: Proteases and cancer biology*, Springer, NY: (pp. 303–323).

Berndt, S., d'Hauterive, S. P., Blacher, S., Pequeux, C., Lorquet, S., Munaut, C., et al. (2006). Angiogenic activity of human chorionic gonadotropin through LH receptor activation on endothelial and epithelial cells of the endometrium. *The FASEB Journal*, *20*, 2630–2632.

Bruyere, F., & Noel, A. (2010). Lymphangiogenesis: In vitro and in vivo models. *The FASEB Journal*, *24*, 8–21.

Burbridge, M. F., Coge, F., Galizzi, J. P., Boutin, J. A., West, D. C., & Tucker, G. C. (2002). The role of the matrix metalloproteinases during in vitro vessel formation. *Angiogenesis*, *5*, 215–226.

Chabottaux, V., & Noel, A. (2007). Breast cancer progression: Insights into multifaceted matrix metalloproteinases. *Clinical and Experimental Metastasis*, *24*, 647–656.

Cheng, X. W., Kuzuya, M., Nakamura, K., Maeda, K., Tsuzuki, M., Kim, W., et al. (2007). Mechanisms underlying the impairment of ischemia-induced neovascularization in matrix metalloproteinase 2-deficient mice. *Circulation Research*, *100*, 904–913.

Chun, T. H., Sabeh, F., Ota, I., Murphy, H., McDonagh, K. T., Holmbeck, K., et al. (2004). MT1-MMP-dependent neovessel formation within the confines of the three-dimensional extracellular matrix. *Journal of Cell Biology*, *167*, 757–767.

Coussens, L. M., Hanahan, D., & Arbeit, J. M. (1996). Genetic predisposition and parameters of malignant progression in K14-HPV16 transgenic mice. *The American Journal of Pathology*, *149*, 1899–1917.

Coussens, L. M., Tinkle, C. L., Hanahan, D., & Werb, Z. (2000). MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell*, *103*, 481–490.

Egeblad, M., & Werb, Z. (2002). New functions for the matrix metalloproteinases in cancer progression. *Nature Reviews Cancer*, *2*, 161–174.

Fanjul-Fernandez, M., Folgueras, A. R., Cabrera, S., & Lopez-Otin, C. (2009). Matrix metalloproteinases: Evolution, gene regulation and functional analysis in mouse models. *Biochimica et Biophysica Acta*, *1803*, 3–19.

Fluck, M. M., & Schaffhausen, B. S. (2009). Lessons in signaling and tumorigenesis from polyomavirus middle T antigen. *Microbiology and Molecular Biology Reviews*, *73*, 542–563, Table of Contents.

Gomis-Ruth, F. X. (2009). Catalytic domain architecture of metzincin metalloproteases. *The Journal of Biological Chemistry*, *284*, 15353–15357.

Jensen, L. D., Cao, R., & Cao, Y. (2009). In vivo angiogenesis and lymphangiogenesis models. *Current Molecular Medicine*, *9*, 982–991.

Jost, M., Folgueras, A. R., Frerart, F., Pendas, A. M., Blacher, S., Houard, X., et al. (2006). Earlier onset of tumoral anglogenesis in matrix metalloproteinase-19-deficient mice. *Cancer Research*, *66*, 5234–5241.

Jost, M., Vosseler, S., Blacher, S., Fusenig, N. E., Mueller, M. M., & Noel, A. (2008). Chapter 17: The surface transplantation model to study the tumor-host interface. In D. R. Edwards, G. Hoyer-Hansen, F. Blasi, & B. F. Sloane (Eds.), *The cancer degradome: proteases and cancer biology*, Springer, NY: (pp. 327–342).

Lederle, W., Hartenstein, B., Meides, A., Kunzelmann, H., Werb, Z., Angel, P., et al. (2009). MMP13 as a stromal mediator in controlling persistent angiogenesis in skin carcinoma. *Carcinogenesis*, *31*, 1175–1184.

Lin, E. Y., Jones, J. G., Li, P., Zhu, L., Whitney, K. D., Muller, W. J., et al. (2003). Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *The American Journal of Pathology*, *163*, 2113–2126.

London, C. A., Sekhon, H. S., Arora, V., Stein, D. A., Iversen, P. L., & Devi, G. R. (2003). A novel antisense inhibitor of MMP-9 attenuates angiogenesis, human prostate cancer cell invasion and tumorigenicity. *Cancer Gene Therapy*, *10*, 823–832.

Lopez-Otin, C., & Matrisian, L. M. (2007). Emerging roles of proteases in tumour suppression. *Nature Reviews Cancer*, *7*, 800–808.

Lopez-Otin, C., & Overall, C. M. (2002). Protease degradomics: A new challenge for proteomics. *Nature Reviews Molecular Cell Biology*, *3*, 509–519.

Maquoi, E., Sounni, N. E., Devy, L., Olivier, F., Frankenne, F., Krell, H. W., et al. (2004). Anti-invasive, antitumoral, and antiangiogenic efficacy of a pyrimidine-2,4,6-trione derivative, an orally active and selective matrix metalloproteinases inhibitor. *Clinical Cancer Research*, *10*, 4038–4047.

Martin, M. D., Carter, K. J., Jean-Philippe, S. R., Chang, M., Mobashery, S., Thiolloy, S., et al. (2008). Effect of ablation or inhibition of stromal matrix metalloproteinase-9 on lung metastasis in a breast cancer model is dependent on genetic background. *Cancer Research*, *68*, 6251–6259.

Martin, M. D., & Matrisian, L. M. (2007). The other side of MMPs: Protective roles in tumor progression. *Cancer and Metastasis Reviews*, *26*, 717–724.

Masson, V., de la Ballina, L. R., Munaut, C., Wielockx, B., Jost, M., Maillard, C., et al. (2005). Contribution of host MMP-2 and MMP-9 to promote tumor vascularization and invasion of malignant keratinocytes. *The FASEB Journal*, *19*, 234–236.

Mott, J. D., & Werb, Z. (2004). Regulation of matrix biology by matrix metalloproteinases. *Current Opinion in Cell Biology*, *16*, 558–564.

Mueller, M. M., & Fusenig, N. E. (2004). Friends or foes – bipolar effects of the tumour stroma in cancer. *Nature Reviews Cancer*, *4*, 839–849.

Nicosia, R. (2009). The aortic ring model of angiogenesis: A quarter century of search and discovery. *Journal of Cellular and Molecular Medicine*, *13*, 4113–4136.

Nielsen, B. S., Egeblad, M., Rank, F., Askautrud, H. A., Pennington, C. J., Pedersen, T. X., et al. (2008). Matrix metalloproteinase 13 is induced in fibroblasts in polyomavirus middle T antigen-driven mammary carcinoma without influencing tumor progression. *PLoS One*, *3*, e2959.

Noel, A., Jost, M., & Maquoi, E. (2008). Matrix metalloproteinases at cancer tumor-host interface. *Seminars in Cell and Developmental Biology*, *19*, 52–60.

Nozawa, H., Chiu, C., & Hanahan, D. (2006). Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 12493–12498.

Olson, E. S., Aguilera, T. A., Jiang, T., Ellies, L. G., Nguyen, Q. T., Wong, E. H., et al. (2009). In vivo characterization of activatable cell penetrating peptides for targeting protease activity in cancer. *Integrative biology(Cambridge)*, *1*, 382–393.

Otrock, Z. K., Hatoum, H. A., Awada, A. H., Ishak, R. S., & Shamseddine, A. I. (2009). Hypoxia-inducible factor in cancer angiogenesis: Structure, regulation and clinical perspectives. *Critical Reviews in Oncology/Hematology*, *70*, 93–102.

Overall, C. M. (2004). Dilating the degradome: Matrix metalloproteinase 2 (MMP-2) cuts to the heart of the matter. *Biochemical Journal*, *383*, 413–418.

Page-McCaw, A., Ewald, A. J., & Werb, Z. (2007). Matrix metalloproteinases and the regulation of tissue remodelling. *Nature Reviews Molecular Cell Biology*, *8*, 221–233.

Parangi, S., O'Reilly, M., Christofori, G., Holmgren, L., Grosfeld, J., Folkman, J., et al. (1996). Antiangiogenic therapy of transgenic mice impairs de novo tumor growth. *Proceedings of the National Academy of Sciences of the United States of America*, *93*, 2002–2007.

Rhee, J. S., Diaz, R., Korets, L., Hodgson, J. G., & Coussens, L. M. (2004). TIMP-1 alters susceptibility to carcinogenesis. *Cancer Research*, *64*, 952–961.

Ribatti, D., Nico, B., Crivellato, E., Roccaro, A. M., & Vacca, A. (2007). The history of the angiogenic switch concept. *Leukemia*, *21*, 44–52.

Roy, R., Yang, J., & Moses, M. A. (2009). Matrix metalloproteinases as novel biomarkers and potential therapeutic targets in human cancer. *Journal of Clinical Oncology*, *27*, 5287–5297.

Skobe, M., Rockwell, P., Goldstein, N., Vosseler, S., & Fusenig, N. E. (1997). Halting angiogenesis suppresses carcinoma cell invasion. *Nature Medicine*, *3*, 1222–1227.

Sounni, N. E., Roghi, C., Chabottaux, V., Janssen, M., Munaut, C., Maquoi, E., et al. (2004). Up-regulation of vascular endothelial growth factor-A by active membrane-type 1 matrix metalloproteinase through activation of Src-tyrosine kinases. *Journal of Biological Chemistry*, *279*, 13564–13574.

Szabova, L., Chrysovergis, K., Yamada, S. S., & Holmbeck, K. (2008). MT1-MMP is required for efficient tumor dissemination in experimental metastatic disease. *Oncogene*, *27*, 3274–3281.

Tlsty, T. D. (2001). Stromal cells can contribute oncogenic signals. *Seminars in Cancer Biology*, *11*, 97–104.

Tlsty, T. D., & Coussens, L. M. (2006). Tumor stroma and regulation of cancer development. *Annual Review of Pathology*, *1*, 119–150.

Wojtowicz-Praga, S. (1999). Clinical potential of matrix metalloprotease inhibitors. *Drugs in R&D*, *1*, 117–129.

Yana, I., Sagara, H., Takaki, S., Takatsu, K., Nakamura, K., Nakao, K., et al. (2007). Crosstalk between neovessels and mural cells directs the site-specific expression of MT1-MMP to endothelial tip cells. *Journal of Cell Science*, *120*, 1607–1614.

Zhu, W. H., Guo, X., Villaschi, S., & Francesco Nicosia, R. (2000). Regulation of vascular growth and regression by matrix metalloproteinases in the rat aorta model of angiogenesis. *Laboratory Investigation*, *80*, 545–555.

Chapter 7
Tumor-Stroma Interactions: Focus on Fibroblasts

Donald Gullberg and Rolf K. Reed

Abstract Despite improved diagnosis and therapies, including chemo- and radiotherapy, cancer still is among the leading causes of death in Europe and the world. Not only the tumor itself, but also the stromal tumor microenvironment has recently been pinpointed as a crucial player in tumor progression. Among the molecules of the tumor stroma, extracellular matrix components, integrins, matrix metalloproteinases and cytokines are considered potential targets in therapy approaches aimed at eradicating the tumor.

List of Abbreviations

CAFs	Carcinoma-Associated Fibroblasts
desmoplasia	Growth of Fibrous Connective Tissue
EMT	Epithelial Mesenchymal Transition
ECM	Extracellular Matrix
MET	Mesenchymal Epithelial Transition
MPPs	Matrix Metalloproteinases
SIBLING	Small Integrin-Binding Ligand N-Linked Glycoprotein

7.1 Introduction

Cancer is the second leading cause of death in Europe (Micheli et al., 2002). Despite higher survival rates due to better diagnosis and improved treatments, there is still an urgent need for novel comprehensive therapeutic approaches as well as n increased understanding of the basic biological principles underlying cancerous transformation and growth.

An epithelial tumor is composed of carcinoma cells and a surrounding tumor stroma. The tumor microenvironment formed by the tumor stroma, contains:

D. Gullberg (✉)
Department of Biomedicine, University of Bergen, Bergen, Norway
e-mail: donald.gullberg@biomed.uib.no

C. Brakebusch, T. Pihlajaniemi (eds.), *Mouse as a Model Organism*,
DOI 10.1007/978-94-007-0750-4_7,

- cells associated with the tumor-associated vasculature,
- various types of inflammatory cells including tumor associated macrophages,
- carcinoma-associated fibroblasts that interact tissue-specifically with each other, the vascular cells, the immune cells and the tumor cells, and
- soluble and insoluble components of the extracellular space.

Historically in the cancer field much of the focus has been on the changes in the cancer cells themselves. However, in recent years it has been realized that in order to understand and treat cancer one has also to take into account the surrounding tumor stroma, which conditions the microenvironment via dynamic bi-directional cellular interactions (Hanahan & Weinberg, 2000). The fibroblasts, which when activated in the tumor stroma are called carcinoma-associated fibroblasts (CAFs), are interesting because they are the cells that produce the connective tissue that is present in and around the tumor. In a recent finding it was noted that the stiffness of the matrix regulates tumor growth, indirectly implying that the fibroblasts in the tumor microenvironment is a major cell type regulating tumor growth (Levental et al., 2009). These data stress the pivotal role of fibroblasts for tumor growth and suggest that new approaches are needed in order to translate this knowledge into new therapeutic strategies.

7.2 Fibroblasts Are Heterogeneous

In normal epithelial tissues, the stromal compartment provides important architectural structural support for the epithelium and the other cells. The normal stroma is composed of orderly structured meshwork of mesenchymal cells (including fibroblasts), blood and lymphatic vessels, and immune cell infiltrates. Fibroblasts are cells of ectomesenchymal or mesodermal origin that reside in every tissue of the body. Sampling of fibroblasts from different locations in the body has revealed that fibroblasts are characterized by a positional code (Rinn, Bondre, Gladstone, Brown & Chang, 2006). In addition, rather than being cells of a defined fixed phenotype, they appear to be heterogeneous due to different origins and plasticity (Sorrell, Baber & Caplan, 2007).

7.3 Stroma Fibroblasts in Tumors Have Multiple Origins

The CAFs in carcinomas are heterogenous and can originate from tissue fibroblasts, adult mesenchymal stem cells, cells formed via epithelial-mesenchymal-transitions (EMTs) or circulating fibrocytes (Haviv, Polyak, Qiu, Hu & Campbell, 2009; Hinz et al., 2007). During metastasis the escaped tumor cells have been suggested to undergo the reverse process, mesenchymal epithelial transition (MET), at the secondary site. Since the EMT process is very transitory and hard to follow over time in tumors, the contribution of EMT to tumorigenesis, invasion and metastasis in different tumors is controversial (Thompson & Williams, 2008). The suggestion that cells that have formed by EMT have stem cell characteristic have increased the interest

in EMT and MET (Polyak & Weinberg, 2009). The different origins of fibroblasts in many ways are similar to the situation in tissue fibrosis where the contribution of EMT and circulating fibrocytes to tissue fibrosis varies with the type of tissue (Faulkner, Szcykalski, Springer & Barnes, 2005; Kisseleva & Brenner, 2008).

7.4 Fibroblasts Are Activated in Tumors

As pointed out, current cancer research emphasizes the importance of the tumor microenvironment for tumorigenesis, tumor progression and metastasis (Franco, Shaw, Strand & Hayward, 2010). As the stroma is activated by the tumor, mechanisms are initiated that support dynamic and active tissue remodeling (Kalluri & Zeisberg, 2006). Unlike during normal wound healing, the activated status of many cells in the tumor stroma persists and has been compared to that of a non-healing wound (Dvorak, 1986). The changes that occur in the stroma environment of the tumor include: induction of fibroblast proliferation; differentiation into myofibroblasts; changes in the amount and arrangement of stromal collagen; angiogenesis; and increased immune and inflammatory cell infiltrates (Fig. 7.1). The differentiation of fibroblasts into contractile, fibrillar collagen producing cells, referred to as myofibroblasts thus occurs both during wound healing, fibrosis and in tumors (Giatromanolaki, Sivridis & Koukourakis, 2007). Not all of the CAFs are activated into myofibroblasts. In some form of tumors the production of stroma is excessive, and is then referred to as the desmoplastic reaction. In some tumor forms the presence of excessive ECM, leading to a stiff tumor tissue is a poor prognostic factor (Apte & Wilson, 2007). The changes in the tumor stroma are also reflected at biochemical level, and at the regulatory levels of gene expression and epigenetic regulation, as revealed by microarray studies comparing organ specific cancer, versus

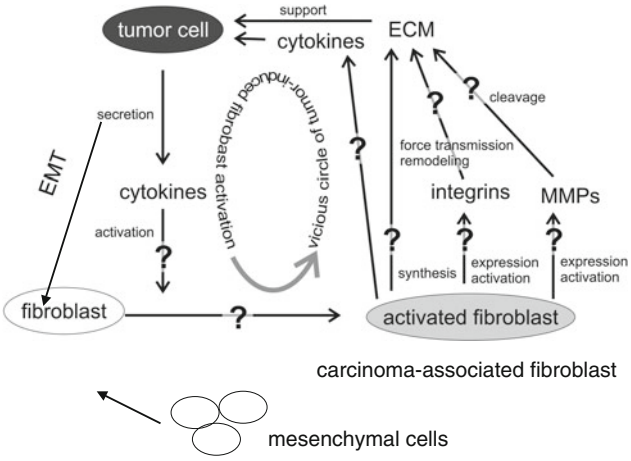

Fig. 7.1 *A schematic illustration of tumor-stroma interactions. Questions marks* denote areas of research where current information is only fragmented. (Picture by courtesy of J. Eble, Univ of Frankfurt.)

normal tissues (Boers et al., 2006; Ma, Dahiya, Richardson, Erlander & Sgroi, 2009; Sadlonova et al., 2009; Sheehan et al., 2008).

We will discuss some of the molecular mechanisms involved in the activation of fibroblasts below.

7.5 Biomarkers for Stroma Fibroblasts and Myofibroblasts

A major challenge within the tumor stroma field is to find good markers for CAFs, and CAF myofibroblasts. A number of proteins are expressed on fibroblasts, but their expression is context-dependent, and usually these biomarkers are expressed on other cell types as well (Table 7.1). A common positive marker used to demarcate

Table 7.1 An overview of some fibroblast markers and their expression pattern. Data accumulated from Pubmed references. Selected references are given

Cells Marker	Fibroblasts	CAFs	CAF-derived myofibroblasts	Fibroblast-specific marker in vivo?	Selected reference(s)
FN -EDA	+	+	+++	*No*, carcinoma cells	Matsumoto et al. (1999) and Pujuguet et al. (1996)
Cadherin-11	+	+	+++	*No*, smooth muscle cells	Monahan et al. (2007)
N-cadherin	++	++	+	*No*, myoepithelial cells, muscle cells, some epithelial cells	Tsuchiya et al. (2006)
Desmin	–	–	+	*No*, muscle cells	Van Muijen et al. (1987)
Vimentin	+	+	+	*No*, lymphocytes, monocytes/ macrophages	Hornbeck et al. (1993) and Nieminen et al. (2006)
α-SMA	–	–	+++	*No*, smooth muscle cells, pericytes	Verbeek et al. (1994)
Integrin α11	+	+	+++	*Yes*	Popova et al. (2007) and Popova et al. (2004)
FSP-1	+	+	+	*No*, immune cells	Cabezon et al. (2007)
FAP	+	+	+	*No*, pericytes	Pure (2009)
Endosialin	+	+	++	*No*, endothelial cells, pericytes	Christian et al. (2008)
PDGFαR	+	+	+	*No*, smooth muscle cells, carcinoma cells	Chaudhry et al. (1992)
Thy-1	+	+	+	*No*, microvascular endothelium	Rege & Hagood (2006)

Abbreviations: FN-EDA: fibronectin splice variant EDA, α-SMA: alpha-smooth muscle actin isoform, FSP-1: fibroblast-specific protein-1, FAP: fibroblast activation protein.

CAFs in tumor tissue is vimentin. For myofibroblasts a contractile form of actin, α-smooth muscle actin (α-SMA), and the ED-A splice variant of fibronectin (FN-EDA) are often used. More recently the fibroblast integrin α11 has been shown to be increased during myofibroblast differentiation (Bystrom, Carracedo, Behndig, Gullberg & Pedrosa-Domellof, 2009; Carracedo et al., 2010) and may therefore be a good candidate for a CAF- and CAF-myofibroblast-specific marker.

However, great care should be used when using markers for fibroblasts, since most markers are not fibroblast-specific. Most cells in the body make one form of collagen or the other and contain prolyl-4-hydroxylase. Although the levels of enzymes involved in collagen biosynthesis are usually high in mesenchymal cells with a high turnover of fibrillar collagens, enzymes involved in synthesis of collagens are by themselves no good markers for fibroblasts. Likewise it is worth noting that fibroblast specific protein-1 (FSP-1) and fibroblasts activation protein (FAP) are both expressed on cell types other than fibroblasts which are abundantly present in the tumor stroma (Cabezon et al., 2007; Pure, 2009). Much of what has been published in this area runs the risk of being "muddy" if one does not perform immune labelling using the appropriate markers to monitor smooth muscle cells, endothelial cells, fibrocytes, stem cells, pericytes and different classes of immune cells. Ultimately the issue about fibroblast markers boils down to the definition of a fibroblast. Remaining issues that are still not resolved include: At what stage does a mesenchymal cell become a fibroblast? Can fibroblasts originate from other embryonic structures than mesodern and ectomesenchyme? In the future we might be able establish transcriptional profiles of developmental stage-, tissue-, and differentiation stage -specific fibroblast identities. Most likely no single protein marker can be used for these different stages, but a combination of markers which in a context-dependent manner can serve to define the different states.

7.6 Fibroblasts Affect Tumor Growth

Studies in the past decade have indicated that fibroblasts activation in the stroma is not merely a bystander phenomenon. Instead the CAFs provide a tumor permissive microenvironment and, therefore, play major roles in the process of tumor progression, including tumor cell growth, invasion and motility, metastasis, tumor angiogenesis, but also chemo/radio resistance (Franco et al., 2009). Exiting new data indicate that fibroblast might play a leading role in enabling cancer cell invasion (Gaggioli et al., 2007) and to be involved in conditioning the pre-metastatic niche (Kaplan, Psaila & Lyden, 2007). Some of the molecular and cellular mechanisms of these processes have started to become unraveled. In additions to paracrine and integrin-mediated MMP-dependent events new data strongly suggest that tumor stiffness, in a process involving mechanical sensing and tensional homeostasis, regulates tumor growth (Levental et al., 2009). Some examples of molecular mechanisms involved in tumor -stroma interactions will be given below.

7.7 Molecular Mechanisms of Tumor-Stroma Interactions

How exactly the tumor stroma interacts with the tumor is an intricate question that is likely to continue and generate data for many years to come. It seems clear hat these interactions are dependent on the type of tumor and the type of tissue- and tumor-specific microenvironment. Extracellular matrix (ECM), integrins, matrix metalloproteinases (MMPs) and growth factors/cytokines are increasingly being recognized as being important in the control of these interactions involving the tumor microenvironment (Radisky, Muschler & Bissell, 2002).

7.7.1 ECM

The normal function of the ECM in a tissue in homeostasis is that it ensures the structural integrity of normal tissue architecture and creates a certain microenvironment with storage of growth factors and proteolytic enzymes. In dynamics situation like tissue regeneration, wound healing and tumor growth the matrix assumes new roles. As part of the host response to the tissue CAFs produce a fibrillar matrix in a desmoplastic reaction. As already mentioned in some forms of cancer this abundant matrix is a prognostic factor, generally serving as a poor prognostic factor (Caporale et al., 2001; Tsujino et al., 2007).

During tumor metastasis, critical events such as basement membrane degradation and establishment of an invasive front, are intimately linked with extracellular matrix function in metastasis initiation (De Wever, Demetter, Mareel & Bracke, 2008). During this phase of tumor spread, ECM components are instructive in cell migratory events in and out of the tumor tissue.

Due to defective lymphatics and leaky blood vessels the interstitial tissue pressure is higher inside the tumor compared to the surrounding tissue. The myofibroblasts contribute to the stroma ECM synthesis and via cell-matrix contractions are thought to contract the tumor stroma and in this way contribute to the higher interstitial fluid pressure (Heldin, Rubin, Pietras & Ostman, 2004). The role of tumor desmoplasia is often underestimated in various xenograft models, and in general orthotopic models seem to be preferred (Takemura, Yashiro, Sunami, Tendo & Hirakawa, 2004).

Recently, experimental modulation of the fibroblast produced fibrillar matrix stiffness was shown to regulate integrin signaling and tumor growth (Levental et al., 2009; Ng & Brugge, 2009). The regulatory role of matrix stiffness and the mechanical strain is another good example of mechanism where tumor desmoplasia shares molecular mechanisms with fibrotic mechanisms (De Wever et al., 2008; Hinz, 2009). Mechanical strain is a factor regulating myofibroblasts differentiation, and plays a similar role in the tumor stroma (Hinz, 2007; Wipff & Hinz, 2009). A major function of mechanical strain in this setting might be to regulate TGF-β activation in an integrin -dependent manner (Hynes, 2009; Margadant and

Sonnenberg, 2010). In addition to the fibrillar collagens which are abundant in the desmoplastic stroma, changes in a number of other matrix proteins have been noted in tumors. Tenascin is a family of fours member, tenascin-C, -X, -W and -R. Tenascin-C and -W are both found in the tumor stroma, and recently tenascin-C has been linked to tumor metastasis (Brellier, Tucker & Chiquet-Ehrismann, 2009). Osteopontin is part of a family of proteins named SIBLINGs, which appear to be important modulators of tumor growth (Bellahcene, Castronovo, Ogbureke, Fisher & Fedarko, 2008). Osteopontin has pro-angiogenic properties (Cui et al., 2007), but was also suggested to contribute to bone-specific metastasis, by a similar αvβ3 integrin- dependent mechanism as that suggested to operate for another SIBLING member, bone specific protein (Bellahcene et al., 2008).

Further attesting to the role of the extracellular matrix is the effect of altered ECM in mice where the small leucin rich protein (SLRP) proteoglycan fibromodulin had been deleted (Oldberg et al., 2007). Fibromodulin is important for the assembly and structure of the collagen fibers. In several implanted tumors, fibromodulin was present in the tumor matrix, although it was absent in normal murine skin. In the fibromodulin-null mice, the human KAT-4 tumors were larger, but displayed an altered collagen structure with thinner and scarcer fibers. Fibromodulin-deficiency did not affect vasculature. However, it raised the extracellular fluid volume and lowered the interstitial pressure in the tumors, likely via an effect that alters the transport properties of the tumor matrix with altered fluid convection inside and out of the stroma.

7.7.2 Integrins

The role of integrins in tumors has mainly focused on integrins on tumor cells and on cells taking part in tumor angiogenesis. Integrins on tumor cells regulate tumor growth, cell migration and resistance to apoptosis in a complex manner involving cross-talk with growth factor receptors (reviewed by Desgrosellier and Cheresh).

A recent article has highlighted the importance of integrin recycling for tumor invasion. Analyzing p53 mutants revealed a series of gain of function mutations that could be shown to facilitate the interaction between rab coupling protein and integrin α5β1 in a complex with EGF receptor (EGFR), facilitating increased endocytotic recycling of EGFR and α5β1 integrin. This increased integrin recycling in turn was observed to lead to increased invasion in experimental models (Muller et al., 2009; Selivanova & Ivaska, 2009).

During the last decade a major focus has been on inhibiting integrin function during tumor angiogenesis. The emerging picture is complex, implicating αv integrins, with conflicting data in different experimental systems (Astrof & Hynes, 2009; Cheresh & Stupack, 2008; Hynes, 2007). In addition, the collagen-binding integrins α1β1 and α2β1(Senger et al., 1997), as well as the finbronectin receptor α5β1 integrin (Astrof & Hynes, 2009), have all been implicated in angiogenesis, and most likely also contribute to tumor angiogenesis, to various degrees. The

collagen-binding integrins seem to work in part via mechanisms regulating MMP expression (Pozzi et al., 2000).

In comparison to the extensive work on integrin expression and function in tumor cells and tumor vasculature, relatively little attention has been given to the role of integrins in the stromal fibroblasts. Some recent studies suggest that this might change and that the stroma fibroblasts will be recognized as major therapeutic targets in the tumor stroma.

In an in vitro model for squamous cell carcinomas, the α3β1 and α5β1 integrins in a 3D microenvironment created by a mixture of matrigel and collagen I, were found to lead the way for carcinoma cells by generating the migration tracks (Gaggioli et al., 2007). Further studies have suggested that a CAF cell line used α5β1 in a Rab21-dependent manner to regulate matrix contraction and remodeling needed for carcinoma invasion (Hooper, Gaggioli & Sahai, 2010). These data are pertinent for the complex activities at the invading front of carcinomas. It seems likely that CAFs in different tissues have distinct integrin repertoires. It will be of interest to determine if the Rab21-dependent mechanisms are shared by the other rab21- binding integrin chains such as the collagen-binding α1 and α11 integrin chains, which both bind Rabb21 (Pellinen et al., 2006).

In a recent study we showed that integrin α11, which is abundantly expressed in the lung tumor stroma in a mouse heterotypic xenograft model, contributed in a paracrine mode to growth of xenografted tumors of A549 cells (Zhu et al., 2007). More recently we have shown that α11 is regulated by mechanical strain, it is up-regulated inside a strained 3D environment, and is functionally important for myofibroblast differentiation (Carracedo et al., 2010). The role of α11β1 in the desmoplastic reaction is, however, not known.

7.7.3 Growth Factors

Previous work has implicated different PDGF isoforms and VEGF in bi-directional signalling between tumor and stroma cells (Andrae, Gallini & Betsholtz, 2008; Dong et al., 2004). In elegant genetic tumor models, both tumor produced PDGF-AA and PDGF-CC were found to stimulate stromal fibroblasts recruitment. Detailed analysis of the PDGF α receptor expressing CAFs revealed a heterogeneous expression of the biomarker FSP-1, suggesting that the target population for PDGF signalling is heterogeneous. A major challenge in the future will be to identify the stromal CAF subpopulation which is responsible for stimulating tumorigenesis. TGF-β plays a complex role in tumor growth with different role during different stages of tumor growth and in addition has major effects on the immune system. In the stroma, TGF-β regulates matrix synthesis, EMT and the behaviour of the stromal fibroblasts (Prud'homme, 2007). Again, attesting to the role of growth factors and an altered extracellular matrix for the tumor biology, inhibitions of PDGF-β receptors attenuates the interstitial hypertension in experimental tumors (Pietras et al., 2001).

Other examples of paracrine signalling include fibroblast-produced SDF-1 which stimulates breast tumorigenesis indirectly via supporting angiogenesis and CCL5 secretion by recruited mesenchymal stem cells, in turn promoting the metastasis of the carcinoma cells (Karnoub et al., 2007). The latter study is controversial since it suggests that metastasizing capacity is mainly dependent on a re-training of the tumor cells by soluble stroma-derived factors (Gullberg, 2008). In some forms of prostate cancer the secretion of sonic hedgehog by the carcinoma result in an altered transcriptional response to Heddgehog in the stroma that mimics the growth-promoting actions of the fetal mesenchyme (Shaw, Gipp & Bushman, 2009).

7.7.4 MMPs

Like the integrin family, the MMP family is a large family with more than 20 members. Both tumor and stroma cells produce different repertoires of MMPs, and their atcivity is further regulated by tumor-stroma interactions. Recent studies present compelling evidence that stroma expression of MMP-13 is required for melanoma invasion and metastasis, partly due to its stimulatory role on angiogenesis (Zigrino et al., 2009). Other studies stress the importance of the membrane bound MMP-14 in tumor spread (Sabeh, Li, Saunders, Rowe & Weiss, 2009a; Sabeh, Shimizu-Hirota & Weiss, 2009b).

In general, MMPs can stimulate or inhibit tumor growth, angiogenesis and invasion/metastasis. These varying and diverse effects depend on the cell type, timing and localization of MMP expression in the tumor. In addition to degrading basement membranes to allow for invasion, major modes of MMP action involve mechanisms regulating growth factor bioavailability and matrix degradation to generate fragments with anti-angiogenesis effects (Barczyk, Carracedo & Gullberg, 2010; Suhr, Brixius & Bloch, 2009). With our current knowledge about the complex role of the large MMP family, it is not surprising that broad-spectrum MMP inhibitors have been shown to be inefficient in a clinical setting.

7.8 Fibroblasts as Anti-cancer Targets

In addition to the molecular and cell biological understanding of the role of CAFs, their role as determinants and/or modulators of treatment responses for chemo- or radiotherapy becomes increasingly evident (Nam, Chung, Hsu & Park, 2009; Zutter, 2007). Given the central role of CAFs in tumor progression and tumor invasion, it is therefore of great interest to target these cells in new approaches. Strategies aimed at reducing tumor stiffness and interstitial tumor pressure will increase the efficiency of combinatory chemotherapy regimens. A few successful examples of this strategy exist and more are likely to be developed along similar principles.

7.9 Future Directions

As the exciting and challenging field of tumor-stroma interactions develops, many basic questions and issues remain to be addressed and answered. For future work it is important to use multiple markers to best define the stromal subpopulation that is being studied.

- A major challenge will be to develop (tumor) specific fibroblast markers and by comparing markers and transcriptional profiling in different type of tumors develop a fibroblast signature for different tumors.
- In experimental studies it will be important to develop a truly fibroblast-specific Cre-mouse strains for fibroblast-specific conditional deletion of genes in a cell-specific manner in the tumor stroma.
- It will be important to continue and develop blocking reagents directed to fibroblasts that decrease interstitial tumor tissue pressure since the interstitial hypertension is considered an obstacle to current therapies. Treatment modalities targeting the fibroblasts and the matrix are therefore predicted to increase the efficacy of chemotherpay.

References

Andrae, J., Gallini, R., & Betsholtz, C. (2008). Role of platelet-derived growth factors in physiology and medicine. *Genes & Development*, *22*, 1276–1312.

Apte, M. V., & Wilson, J. S. (2007). The desmoplastic reaction in pancreatic cancer: An increasingly recognised foe. *Pancreatology*, *7*, 378–379.

Astrof, S., & Hynes, R. O. (2009). Fibronectins in vascular morphogenesis. *Angiogenesis*, *12*, 165–175.

Barczyk, M., Carracedo, S., & Gullberg, D. (2010). Integrins. *Cell and Tissue Research*, *339*, 269–280.

Bellahcene, A., Castronovo, V., Ogbureke, K. U., Fisher, L. W., & Fedarko, N. S. (2008). Small integrin-binding ligand N-linked glycoproteins (SIBLINGs): Multifunctional proteins in cancer. *Nature Reviews Cancer*, *8*, 212–226.

Boers, W., Aarrass, S., Linthorst, C., Pinzani, M., Elferink, R., & Bosma, P. (2006). Transcriptional profiling reveals novel markers of liver fibrogenesis: Gremlin and insulin-like growth factor-binding proteins. *Journal of Biological Chemistry*, *281*, 16289–16295.

Brellier, F., Tucker, R. P., & Chiquet-Ehrismann, R. (2009). Tenascins and their implications in diseases and tissue mechanics. *Scandinavian Journal of Medicine and Science in Sports*, *19*, 511–519.

Bystrom, B., Carracedo, S., Behndig, A., Gullberg, D., & Pedrosa-Domellof, F. (2009). Alpha11 integrin in the human cornea: Importance in development and disease. *Investigative Ophthalmology & Visual Science*, *50*, 5044–5053.

Cabezon, T., Celis, J. E., Skibshoj, I., Klingelhofer, J., Grigorian, M., Gromov, P., et al. (2007). Expression of S100A4 by a variety of cell types present in the tumor microenvironment of human breast cancer. *International Journal of Cancer*, *121*, 1433–1444.

Caporale, A., Cosenza, U. M., Vestri, A. R., Giuliani, A., Costi, U., Galati, G., et al. (2001). Has desmoplastic response extent protective action against tumor aggressiveness in gastric carcinoma? *Journal of Experimental and Clinical Cancer Research*, *20*, 21–24.

Carracedo, S., Lu, N., Popova, S. N., Jonsson, R., Eckes, B., & Gullberg, D. (2010) The fibroblast integrin {alpha}11{beta}1 is induced in a mechanosensitive manner involving activin A and regulates myofibroblast differentiation. *Journal of Biological Chemistry*, *285*, 10434–10445.

Chaudhry, A., Papanicolaou, V., Oberg, K., Heldin, C. H., & Funa, K. (1992). Expression of platelet-derived growth factor and its receptors in neuroendocrine tumors of the digestive system. *Cancer Research*, *52*, 1006–1012.

Cheresh, D. A., & Stupack, D. G. (2008). Regulation of angiogenesis: Apoptotic cues from the ECM. *Oncogene*, *27*, 6285–6298.

Christian, S., Winkler, R., Helfrich, I., Boos, A. M., Besemfelder, E., Schadendorf, D., et al. (2008). Endosialin (tem1) is a marker of tumor-associated myofibroblasts and tumor vessel-associated mural cells. *American Journal of Pathology*, *172*, 486–494.

Cui, R., Takahashi, F., Ohashi, R., Gu, T., Yoshioka, M., Nishio, K., et al. (2007). Abrogation of the interaction between osteopontin and alphavbeta3 integrin reduces tumor growth of human lung cancer cells in mice. *Lung Cancer*, *57*, 302–310.

De Wever, O., Demetter, P., Mareel, M., & Bracke, M. (2008). Stromal myofibroblasts are drivers of invasive cancer growth. *International Journal of Cancer*, *123*, 2229–2238.

Desgrosellier, J. S., & Cheresh, D. A. Integrins in cancer: Biological implications and therapeutic opportunities. *Nature Reviews Cancer*, *10*, 9–22.

Dong, J., Grunstein, J., Tejada, M., Peale, F., Frantz, G., Liang, W. C., et al. (2004). VEGF-null cells require PDGFR alpha signaling-mediated stromal fibroblast recruitment for tumorigenesis. *The EMBO Journal*, *23*, 2800–2810.

Dvorak, H. F. (1986). Tumors: Wounds that do not heal. Similarities between tumor stroma generation and wound healing. *The New England Journal of Medicine*, *315*, 1650–1659.

Faulkner, J. L., Szcykalski, L. M., Springer, F., & Barnes, J. L. (2005). Origin of interstitial fibroblasts in an accelerated model of angiotensin II-induced renal fibrosis. *The American Journal of Pathology*, *167*, 1193–1205.

Franco, O. E., Shaw, A. K., Strand, D. W., & Hayward, S. W. (2010). Cancer associated fibroblasts in cancer pathogenesis. *Seminars in Cell and Developmental Biology*, *21*, 33–39.

Gaggioli, C., Hooper, S., Hidalgo-Carcedo, C., Grosse, R., Marshall, J. F., Harrington, K., et al. (2007). Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. *Nature Cell Biology*, *9*, 1392–1400.

Giatromanolaki, A., Sivridis, E., & Koukourakis, M. I. (2007). The pathology of tumor stromatogenesis. *Cancer Biology & Therapy*, *6*, 639–645.

Gullberg, D. (2008). How to keep that stemmy-ness: Stem cells in the spotlight. *Matrix Biology*, *27*, 161–162.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, *100*, 57–70.

Haviv, I., Polyak, K., Qiu, W., Hu, M., & Campbell, I. (2009). Origin of carcinoma associated fibroblasts. *Cell Cycle*, *8*, 589–595.

Heldin, C. H., Rubin, K., Pietras, K., & Ostman, A. (2004). High interstitial fluid pressure – An obstacle in cancer therapy. *Nature Reviews Cancer*, *4*, 806–813.

Hinz, B. (2007). Formation and function of the myofibroblast during tissue repair. *The Journal of Investigative Dermatology*, *127*, 526–537.

Hinz, B. (2009). Tissue stiffness, latent TGF-beta1 activation, and mechanical signal transduction: Implications for the pathogenesis and treatment of fibrosis. *Current Rheumatology Reports*, *11*, 120–126.

Hinz, B., Phan, S. H., Thannickal, V. J., Galli, A., Bochaton-Piallat, M. L., & Gabbiani, G. (2007). The myofibroblast: One function, multiple origins. *The American Journal of Pathology*, *170*, 1807–1816.

Hooper, S., Gaggioli, C., & Sahai, E. (2010). A chemical biology screen reveals a role for Rab21-mediated control of actomyosin contractility in fibroblast-driven cancer invasion. *British Journal of Cancer*, *102*, 392–402.

Hornbeck, P. V., Garrels, J. I., Capetanaki, Y., & Heimer, S. (1993). Vimentin expression is differentially regulated by IL-2 and IL-4 in murine T cells. *Journal of Immunology*, *151*, 4013–4021.

Hynes, R. O. (2007). Cell-matrix adhesion in vascular development. *Journal of Thrombosis and Haemostasis*, *5*(Suppl 1), 32–40.

Hynes, R. O. (2009). The extracellular matrix: Not just pretty fibrils. *Science.*, *326*, 1216–1219.

Kalluri, R., & Zeisberg, M. (2006). Fibroblasts in cancer. *Nature Reviews Cancer*, *6*, 392–401.

Kaplan, R. N., Psaila, B., & Lyden, D. (2007). Niche-to-niche migration of bone-marrow-derived cells. *Trends in Molecular Medicine*, *13*, 72–81.

Karnoub, A. E., Dash, A. B., Vo, A. P., Sullivan, A., Brooks, M. W., Bell, G. W., et al. (2007). Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature*, *449*, 557–563.

Kisseleva, T., & Brenner, D. A. (2008). Mechanisms of fibrogenesis. *Experimental Biology and Medicine (Maywood)*, *233*, 109–122.

Levental, K. R., Yu, H., Kass, L., Lakins, J. N., Egeblad, M., Erler, J. T., et al. (2009). Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell*, *139*, 891–906.

Ma, X., Dahiya, S., Richardson, E., Erlander, M., & Sgroi, D. (2009). Gene expression profiling of tumor microenvironment during breast cancer progression. *Breast Cancer Research*, *11*, R7.

Margadant, C., & Sonnenberg, A. (2010). Integrin-TGF-beta crosstalk in fibrosis, cancer and wound healing. *EMBO Reports*, *11*, 97–105.

Matsumoto, E., Yoshida, T., Kawarada, Y., & Sakakura, T. (1999). Expression of fibronectin isoforms in human breast tissue: Production of extra domain A+/extra domain B+ by cancer cells and extra domain A+ by stromal cells. *Japanese Journal of Cancer Research*, *90*, 320–325.

Micheli, A., Mugno, E., Krogh, V., Quinn, M. J., Coleman, M., Hakulinen, T., et al. (2002). Cancer prevalence in European registry areas. *The Annals of Oncology*, *13*, 840–865.

Monahan, T. S., Andersen, N. D., Panossian, H., Kalish, J. A., Daniel, S., Shrikhande, G. V., et al. (2007). A novel function for cadherin 11/osteoblast-cadherin in vascular smooth muscle cells: modulation of cell migration and proliferation. *Journal of Vascular Surgery*, *45*, 581–589.

Muller, P. A., Caswell, P. T., Doyle, B., Iwanicki, M. P., Tan, E. H., Karim, S., et al. (2009). Mutant p53 drives invasion by promoting integrin recycling. *Cell*, *139*, 1327–1341.

Nam, J. M., Chung, Y., Hsu, H. C., & Park, C. C. (2009). beta1 integrin targeting to enhance radiation therapy. *International Journal of Radiation Biology*, *85*, 923–928.

Ng, M. R., & Brugge, J. S. (2009). A stiff blow from the stroma: Collagen crosslinking drives tumor progression. *Cancer Cell*, *16*, 455–457.

Nieminen, M., Henttinen, T., Merinen, M., Marttila-Ichihara, F., Eriksson, J. E., & Jalkanen, S. (2006). Vimentin function in lymphocyte adhesion and transcellular migration. *Nature Cell Biology*, *8*, 156–162.

Pellinen, T., Arjonen, A., Vuoriluoto, K., Kallio, K., Fransen, J. A., & Ivaska, J. (2006). Small GTPase Rab21 regulates cell adhesion and controls endosomal traffic of beta1-integrins. *The Journal of Cell Biology*, *173*, 767–780.

Polyak, K., & Weinberg, R. A. (2009). Transitions between epithelial and mesenchymal states: Acquisition of malignant and stem cell traits. *Nature Reviews Cancer*, *9*, 265–273.

Popova, S. N., Rodriguez-Sanchez, B., Liden, A., Betsholtz, C., Van Den Bos, T., & Gullberg, D. (2004). The mesenchymal alpha11beta1 integrin attenuates PDGF-BB-stimulated chemotaxis of embryonic fibroblasts on collagens. *Developmental Biology*, *270*, 427–442.

Popova, S. N., Barczyk, M., Tiger, C. F., Beertsen, W., Zigrino, P., Aszodi, A., et al. (2007). Alpha11 beta1 integrin-dependent regulation of periodontal ligament function in the erupting mouse incisor. *Molecular and Cellular Biology*, *27*, 4306–4316.

Pozzi, A., Moberg, P. E., Miles, L. A., Wagner, S., Soloway, P., & Gardner, H. A. (2000). Elevated matrix metalloprotease and angiostatin levels in integrin α1 knockout mice cause reduced tumor vascularization. *Proceedings of the National Academy of Sciences of the United States of America*, *97*, 2202–2207.

Prud'homme, G. J. (2007). Pathobiology of transforming growth factor beta in cancer, fibrosis and immunologic disease, and therapeutic considerations. *Laboratory Investigation*, *87*, 1077–1091.

Pujuguet, P., Hammann, A., Moutet, M., Samuel, J. L., Martin, F., & Martin, M. (1996). Expression of fibronectin ED-A+ and ED-B+ isoforms by human and experimental colorectal cancer. Contribution of cancer cells and tumor-associated myofibroblasts. *American Journal of Pathology*, *148*, 579–592.

Pure, E. (2009). The road to integrative cancer therapies: Emergence of a tumor-associated fibroblast protease as a potential therapeutic target in cancer. *Expert Opinion Therapeutic Targets*, *13*, 967–973.

Radisky, D., Muschler, J., & Bissell, M. J. (2002). Order and disorder: The role of extracellular matrix in epithelial cancer. *Cancer Investigation*, *20*, 139–153.

Rege, T. A., & Hagood, J. S. (2006). Thy-1, a versatile modulator of signaling affecting cellular adhesion, proliferation, survival, and cytokine/growth factor responses. *Biochimica et Biophysica Acta*, *1763*, 991–999.

Rinn, J. L., Bondre, C., Gladstone, H. B., Brown, P. O., & Chang, H. Y. (2006). Anatomic demarcation by positional variation in fibroblast gene expression programs. *PLoS Geneicst*, *2*, e119.

Sabeh, F., Li, X., Saunders, T., Rowe, R., & Weiss, S. (2009a). Secreted versus membrane-anchored collagenases: Relative roles in fibroblast-dependent collagenolysis and invasion. *Journal of Biological Chemistry*, *284*, 23001–23011.

Sabeh, F., Shimizu-Hirota, R., & Weiss, S. (2009b). Protease-dependent versus -independent cancer cell invasion programs: Three-dimensional amoeboid movement revisited. *The Journal of Cell Biology*, 185, 11–19.

Sadlonova, A., Bowe, D., Novak, Z., Mukherjee, S., Duncan, V., Page, G., et al. (2009). Identification of molecular distinctions between normal breast-associated fibroblasts and breast cancer-associated fibroblasts. *Cancer Microenvironment, 2*, 9–21.

Selivanova, G., & Ivaska, J. (2009). Integrins and mutant p53 on the road to metastasis. *Cell*, *139*, 1220–1222.

Senger, D. R., Claffey, K. P., Benes, J. E., Perruzzi, C. A., Sergiou, A. P., & Detmar, M. (1997). Angiogenesis promoted by vascular endothelial growth factor: Regulation through α1β1 and α2β1 integrins. *Proceedings of the National Academy of Sciences of the United States of America*, *94*, 13612–13617.

Shaw, A., Gipp, J., & Bushman, W. (2009). The Sonic Hedgehog pathway stimulates prostate tumor growth by paracrine signaling and recapitulates embryonic gene expression in tumor myofibroblasts. *Oncogene*, *28*, 4480–4490.

Sheehan, K. M., Gulmann, C., Eichler, G. S., Weinstein, J. N., Barrett, H. L., Kay, E. W., et al. (2008). Signal pathway profiling of epithelial and stromal compartments of colonic carcinoma reveals epithelial-mesenchymal transition. *Oncogene*, *27*, 323–331.

Sorrell, J. M., Baber, M. A., & Caplan, A. I. (2007). Clonal characterization of fibroblasts in the superficial layer of the adult human dermis. *Cell and Tissue Research*, *327*, 499–510.

Suhr, F., Brixius, K., & Bloch, W. (2009). Angiogenic and vascular modulation by extracellular matrix cleavage products. *Current Pharmaceutical Design*, *15*, 389–410.

Takemura, S., Yashiro, M., Sunami, T., Tendo, M., & Hirakawa, K. (2004). Novel models for human scirrhous gastric carcinoma in vivo. *Cancer Science*, *95*, 893–900.

Thompson, E. W., & Williams, E. D. (2008). EMT and MET in carcinoma—clinical observations, regulatory pathways and new models. *Clinical and Experimental Metastasis*, *25*, 591–592.

Tsuchiya, B., Sato, Y., Kameya, T., Okayasu, I., & Mukai, K. (2006). Differential expression of N-cadherin and E-cadherin in normal human tissues. *Archives of Histology and Cytology*, *69*, 135–145.

Tsujino, T., Seshimo, I., Yamamoto, H., Ngan, C. Y., Ezumi, K., Takemasa, I., et al. (2007). Stromal myofibroblasts predict disease recurrence for colorectal cancer. *Clinical Cancer Research*, *13*, 2082–2090.

Van Muijen, G. N., Ruiter, D. J., & Warnaar, S. O. (1987). Coexpression of intermediate filament polypeptides in human fetal and adult tissues. *Laboratory Investigation*, *57*, 359–369.

Verbeek, M. M., Otte-Holler, I., Wesseling, P., Ruiter, D. J., & de Waal, R. M. (1994). Induction of alpha-smooth muscle actin expression in cultured human brain pericytes by transforming growth factor-beta 1. *American Journal of Pathology*, *144*, 372–382.

Wipff, P. J., & Hinz, B. (2009). Myofibroblasts work best under stress. *Journal of Bodywork and Movement Therapies*, *13*, 121–127.

Zhu, C. Q., Popova, S. N., Brown, E. R., Barsyte-Lovejoy, D., Navab, R., Shih, W., et al. (2007). Integrin alpha 11 regulates IGF2 expression in fibroblasts to enhance tumorigenicity of human non-small-cell lung cancer cells. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 11754–11759.

Zigrino, P., Kuhn, I., Bauerle, T., Zamek, J., Fox, J. W., Neumann, S., et al. (2009). Stromal expression of MMP-13 is required for melanoma invasion and metastasis. *The Journal of Investigative Dermatology*, *129*, 2686–2693.

Zutter, M. M. (2007). Integrin-mediated adhesion: Tipping the balance between chemosensitivity and chemoresistance. *Advances in Experimental Medicine and Biology*, *608*, 87–100.

Chapter 8
Experimental Procedures to Assay Invasion-Associated Activities of Primary Cultured Fibroblasts

An Hendrix, Koen Jacobs, Astrid De Boeck, Wendy Westbroek, Marc Bracke, and Olivier De Wever

Abstract Recruitment of distant and local fibroblast-like cells is a process that is essential for tissue repair. Migration and invasion of such cell types requires cytoskeletal rearrangements and polarized vesicle trafficking. Importantly, deregulated invasive behavior contributes to pathological processes including cancer cell invasion and metastasis. A standardized approach for isolation and culturing of primary fibroblasts from wild type or transgenic mice is essential for consistent cell culture experiments. Our research on the invasive phenotype of primary isolated fibroblasts, prompted us to establish a collection of protocols to assay these functions. The collagen type I invasion assay allows examination of cellular invasion into a three dimensional fibrillar network. Alternatively, a Matrigel transwell assay allows the study of chemo-invasion through reconstituted basement membranes. The monolayer wound healing assay is a convenient and inexpensive method for analysis of two dimensional cell migration. All assays are compatible with imaging of live cells during migration/invasion to monitor intracellular events if desired.

8.1 Introduction

Migration is of great importance for gastrulation and organ formation in developing embryos and, if misregulated, can have dire consequences, for example during cancer metastasis (Hanahan & Weinberg, 2000). In addition, recruitment of distant and local fibroblast-like cells is a process that is essential for tissue repair, invasive tumor growth and formation of the pre-metastatic niche (De Wever et al., 2008; Wels et al., 2008).

Fibroblasts develop pronounced morphological asymmetries during directed migration and invasion. The development of this asymmetry depends on cytoskeletal rearrangements and polarized vesicle trafficking (Schmoranzer et al., 2003). Migration and invasion require endocytic and exocytic vesicle trafficking for the

O. De Wever (✉)
Laboratory of Experimental Cancer Research, Department of Radiation Oncology and Experimental Cancer Research, Ghent University Hospital, 9000 Gent, Belgium
e-mail: olivier.dewever@UGent.be

C. Brakebusch, T. Pihlajaniemi (eds.), *Mouse as a Model Organism*,
DOI 10.1007/978-94-007-0750-4_8,

focalized delivery of proteins and/or lipids, which are needed to facilitate extracellular matrix formation, membrane protrusions and adhesion plaques. For example, integrin-based adhesion, required for single cell migration and tissue morphogenesis, is regulated through endocytic trafficking of integrins from the cell rear to the leading edge in fibroblasts. Recently, β1-integrin endocytosis has been studied in fibroblasts that have been isolated from mice in which the tyrosines of the cytoplasmic tail of β1-integrin (NXXY, X denotes any amino acid) were substituted for phenylalanines. This elegant genetic approach clearly indicated an involvement of the NXXY conserved motifs in β1-integrin endocytosis (Pellinen et al., 2008). Furthermore, fibroblasts undergo constitutive and regulated exocytosis. Constitutive exocytosis is conducted by all cell types and is responsible for the delivery of newly synthesized membrane proteins to the plasma membrane and for the release of extracellular proteins. Regulated exocytosis is a tightly regulated process in which the membranes of cytoplasmic organelles fuse with the plasma membrane in response to stimulation. In specialized cell types, such as neurons and cells of the exocrine, endocrine and immunological system, the process regulates secretion of products (e.g. neurotransmitters, hormones and enzymes) within the lumen of the organelle into the extracellular space (i.e., secretory exocytosis) (Burgoyne & Morgan, 2003). In other cases, as in (myo)fibroblasts, exocytosis functions to transfer the organelle membrane and it components to the cell surface (e.g. during repair of membrane ruptures) (Jaiswal et al., 2002).

Both endocytic and exocytic vesicle trafficking pathways are mastered by Rab GTPases and their effector proteins (e.g. Melanophilin and Rabphilin), SNAREs and their regulators (e.g. syntaxin and VAMPs), and Ca^{2+}-binding proteins such as synaptotagmins and calmodulins (Burgoyne & Morgan, 2003). Regulated exocytosis, is shown to be implicated in lysosome related organelle disorders and cancer progression (Huizing et al., 2008; Hendrix et al., 2010). The presence of natural mouse models and engineered mutant mouse lines provide a large collection of models for in vivo analysis of distinct gene functions. Therefore, a unique mouse clinic for standardized comprehensive phenotyping has been established (Gailus-Durner et al., 2005). In addition, the large mouse collection can be used for the isolation of primary mesenchymal cells (e.g. (myo)fibroblasts and bone marrow-derived mesenchymal stem cells). In vitro analysis of these transgenic cells by complementary cell culture models may assist in the characterization of the mouse phenotype. Several natural mouse models for studying lysosome related organelle disorders exist such as the *dilute* (Myosin 5A), *leaden* (Melanophilin), and *ashen* (Rab27A) models (Huizing et al., 2008). Several knock-out (Rab3, Rab27A, Rab27B, Synaptotagmin VII), double knock-out (Rab27A and Rab27B) and transgenic (dominant negative/constitutive active Rab27A or Rab27B) mouse models are available for studying regulated exocytosis (Geppert et al., 1997; Jaiswal et al., 2002; Ramalho et al., 2002; Mizuno et al., 2007).

The feasibility of performing migration and invasion assays of primary isolated fibroblasts or mesenchymal stem cells from transgenic mice has been demonstrated by several groups. For example, skin fibroblasts lacking early growth response gene (Egr-1) showed reduced wound healing migration (Wu, Melichian et al., 2009).

Deficiency of the LIM-only protein Fhl2 impairs wound healing migration of bone marrow-derived mesenchymal stem cells and skin fibroblasts (Wixler et al., 2007). Disrupted c-Jun expression in mouse embryonic fibroblasts reduces wound healing migration and Matrigel transwell invasion (Jiao et al., 2008).

In this review we provide a collection of experimental procedures to assay invasion. Although most assays are originally developed to study invasion/migration of cancer cells, they actually allow for invasion studies of any cell type, including (myo)fibroblasts.

8.2 Isolation of Primary Cells

Fibroblast are easily isolated and expanded from a variety of tissues to study basic aspects of cell biology as well as genomic, biochemical and functional abnormalities in human patients and in transgenic or knockout animals. Sampling of fibroblasts from different locations in the body has revealed that fibroblasts are characterized by a positional code (Chang et al., 2002). In addition, rather than being cells of a defined fixed phenotype, they appear to be heterogeneous and have a dynamically changing plasticity (Sugimoto et al., 2006). We believe that there exist distinct types of fibroblasts with distinct reaction patterns. The spectrum of phenotypic entities ranges from the non-contractile fibroblast to the contractile α-smooth muscle actin-positive myofibroblast with a number of intermediate phenotypes having been described (reviewed in (Eyden, 2005)).

Two methods are frequently used for the isolation of fibroblasts from tissues: explant culture release and enzymatic tissue dissociation (Hentzer & Kobayasi, 1978; Normand & Karasek, 1995; Wang et al., 2004). The first technique employs an explant culture system in which fibroblasts grow out from tissue specimens. The second technique employs a dissociated fibroblast culture system in which fibroblasts are first released from tissue specimens by enzymatic digestion and then placed in culture. When a primary culture is prepared using both methods, it is often heterogeneous in nature (van den Bogaerdt et al., 2002; Wang et al., 2004). Incubation at 37°C and 10% CO_2 in a humidified incubator in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (Sigma), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Sigma) (i.e., complete growth medium) favors the proliferation of fibroblasts and when cultures reach subconfluence, spindle-like fibroblasts are primarily observed. By lowering the incubation temperature, fibroblastic growth is inhibited (Jensen & Therkelsen, 1981). In general, the explant culture system is technically more simple, requiring almost no special experience or reagents, whereas the dissociated fibroblast culture is more suitable for obtaining relatively large numbers of fibroblasts in a short period (Wang et al., 2004). For both methods the tissue fragments are first dipped in 70% ethanol for 2 s to avoid contamination and rinsed with DMEM supplemented with 10% FCS, 1,000 U ml^{-1} penicillin, and 1,000 μg ml^{-1} streptomycin.

For **explant culture**, the tissue fragments are cut in small pieces (2–3 mm^3) and transferred into a 6-well plate with a rough surface (obtained by scratching the surface with a scalpel). 100 μl of 10% FCS supplemented with 1,000 U ml^{-1} penicillin, and 1,000 μg ml^{-1} streptomycin is added on top of each fragment. The cultures are placed in a humidified 37°C, 10% CO_2 incubator for 24 h. After 24 h, 3 ml of complete growth medium is added into each well. Medium is changed every 3–4 days, taking care not to detach the pieces from the surface. Cell outgrowth is observed after 3–6 days. After 15 days, colonies of fibroblast-like cells have been formed which can be transferred at subconfluence to a 25 cm^2 tissue culture flask by trypsinization with a trypsin-EDTA (0.25%–1 mM) solution. This procedure is demonstrated by wild type versus *beige-J* mice. *Beige-J* mice are a natural mouse model of Chediak-Higashi syndrome, a human disorder characterized by enlarged lysosomes and lysosome-related organelles in many cell types (Huizing et al., 2008). Lysosomes in *beige-J* fibroblasts have impaired lysosomal exocytosis, affecting the wound healing process (Ward et al., 2000; Shiflett et al., 2002). Primary *beige-J* fibroblasts are characterized by enlarged lysosomes in the perinuclear area as evidenced by confocal images of the lysosome specific-marker LAMP1 (lysosome-associated membrane protein) on cultured cells (Fig. 8.1).

Enzymatic tissue dissociation requires the use of enzymes able to degrade the interstitial collagenous tissue without damaging the cells. Enzymatic digestion can be combined with automated dissociation of tissues by using a gentleMACS® dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Collagenase is most frequently used for tissue dissociation. The most potent collagenase is the "crude" collagenase secreted by the anaerobic bacteria *Clostridium histolyticum* (CHC).

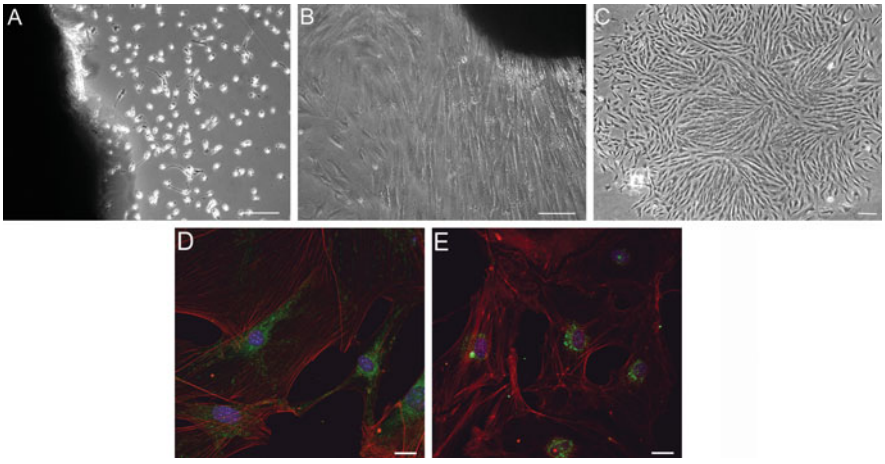

Fig. 8.1 Images of explant culture. After 5 days cell outgrowth can be observed (**a**). After 15 days, colonies of fibroblast-like cells have been formed (**b**). Primary fibroblast culture after 3 weeks (**c**). Scale bar: 100 μm (**d** and **e**) Confocal images of LAMP1, F-actin and DAPI stained fibroblasts from wild type (**d**) and *beige J* (**e**) mice. Scale bar: 20 μm

"Crude" collagenase refers to the fact that the preparation is actually a mixture of several different enzymes besides collagenase that act together to break down tissue. Class I and class II collagenases are present, together with other proteases and enzymes, such as neutral protease, clostripain (a sulfhydryl protease), trypsin, elastase, aminopeptidase, and other polysaccharide- and lipid-degrading enzymes (Matsushita & Okabe, 2001). The overall CHC enzyme composition and activities are very variable between different CHC batches (Wang et al., 2004). Alternatively trypsin, although more cytotoxic than collagenase, can be used for tissue digestion. Tissue pieces (2–3 mm^3) are incubated overnight in 0.2 mg ml^{-1} CHC (type IA; cat no. C9891, Sigma) in serum-free medium. After 24 h, the supernatant is filtered through 100 μm nylon sieves to remove tissue debris. After centrifugation for 10 min at 200 g and 4°C, the supernatant is aspirated and the pellet is resuspended in 100–200 μl of complete growth medium. Cells are counted and 3–10 × 10^4 cells are plated in a 25 cm^2 tissue culture flask in 5 ml of complete growth medium.

8.3 Collagen Type I Invasion

Tissue invasion requires infiltration into an extracellular matrix (ECM) dominated by cross-linked networks of collagen type I. The invasion model, presented here, consists of native, acid-extracted rat tail collagen type I (cat.no. 354236, BD Biosciences, Franklin Lakes, NJ) containing nonhelical telopeptides situated at the N- and C-terminal ends. These telopeptides play an important role in intermolecular covalent cross-links necessary for a gel architecture presenting itself as a structural barrier to cancer cell traffic (Sabeh et al., 2009a). A multicellular spheroid system is more suitable for recreating an in vivo-like environment for migrating cells to study invasion mechanisms. Optimal spheroids are compact with a diameter of 150 μm. The cell number needed to create spheroids of ± 150 μm in diameter has to be determined empirically (Friedrich et al., 2009). In our setup, compact spheroid formation is routinely checked by individual macroscopic evaluation for cell concentrations ranging from 5 × 10^4 to 5 × 10^5 cells ml^{-1}. Single, dissociated cells are diluted to appropriate concentrations in 6 ml complete growth medium in a 50 ml Erlenmeyer flask and incubated on a Gyrotory shaker at 37°C and 70 rpm in a humidified atmosphere with 10% CO_2 in air for 72 h. This cell number is sufficient to obtain +/− 100 compact spheroids of 150 μm diameter. However, rotating culture systems require relatively high quantities of cells, conflicting with the usual demand for minimum amounts of cells when using primary cultures; it may thus be necessary to transfer the cells to stationary spheroid culture systems. Stationary culturing technologies include the growth of spheroid cultures in non-adherent dishes, 96-well plates or as hanging drops (Friedrich et al., 2009).

Collagen type I solution is prepared with a final concentration of 1 mg ml^{-1} collagen type I by mixing the following pre-cooled (stored at 4°C) components: 4 volumes collagen type I (stock is 3.49 mg ml^{-1}), 5 volumes of CMF-HBSS, 1 volume of MEM (10×), 1 volume of 0.25 M $NaHCO_3$, 2.65 volumes of standard medium and 0.3 volumes of 1 M NaOH to make the solution alkaline. Selected,

compact spheroids are mixed with collagen type I solution and gently poured on a preformed bottom gel layer of collagen type I. The experimental set-up is placed at 37°C in a humidified atmosphere with 10% CO_2 in air for at least 1 h. Culture medium is carefully added after gelification (De Wever et al., 2009).

Invasion of unlabeled cells in transparent 3D collagen gels can be monitored by phase contrast microscopy. Alternatively confocal microscopy can be used, after fixation of collagen gels by 3% paraformaldehyde and staining of cells to reveal the organization of the F-actin cytoskeleton (for example staining with Alexa Fluor®-555 phalloidin) (Invitrogen, Carlsbad, Ca) and DAPI (Sigma). The number of invading cells per high powered field and leading front depth of invasion (three or more cells) are quantified as the mean +/− SEM (standard error mean) of at least

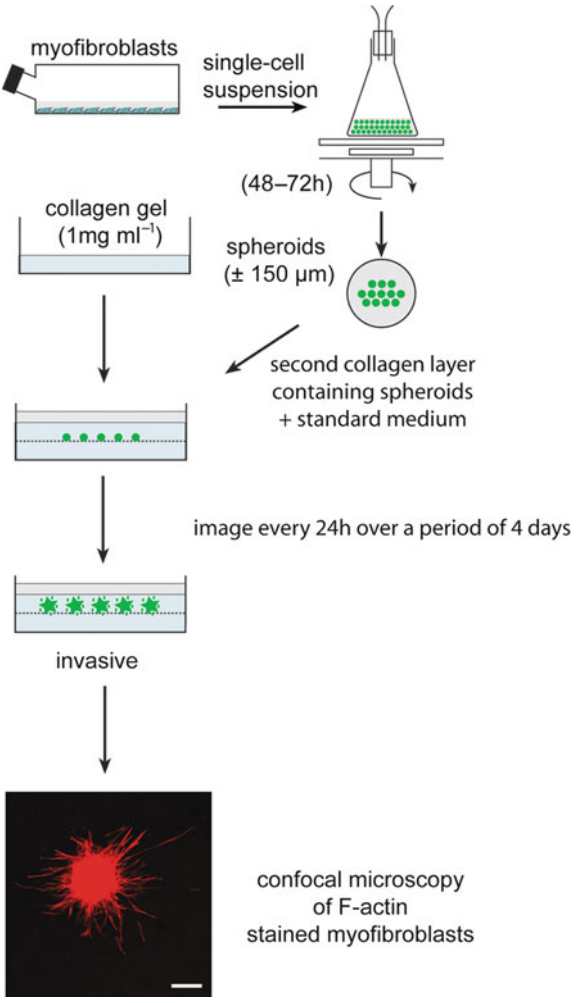

Fig. 8.2 Schematic representation of spheroid collagen type I invasion assay (Myo)fibroblast cultures are trypsinized and a single cell suspension is added to an Erlenmeyer on a Gyrotory shaker during 48–72 h. Compact spheroids are selected and carefully suspended in collagen type I solution. The combined collagen type I-spheroid solution is added on top of a pre-existing collagen type I gel layer. Invasion is followed by phase contrast microscopy during 4 days. At the end of the experiment collagen gels are fixed by paraformaldehyde (3%) and stained by fluorescently labelled phalloidin (Alexa Fluor®-555 phalloidin) to reveal the organization of the F-actin cytoskeleton by confocal microscopy. Scale bar: 100 µm

three experiments (Sabeh et al., 2009b). As shown in Fig. 8.2, myofibroblasts subsequently activate a tissue-invasive program and infiltrate the surrounding ECM in a "starburst" pattern. Cell density at the surface of the spheroid appears to decrease, suggesting that a resident population of myofibroblast spheroid cells invades the gel, rather than only new cells generated by cell proliferation. When myofibroblasts invade the collagen, a reorganization of the collagen into thick collagen fibers (straps) that align parallel to the axes between spheroids may occur (Denys et al., 2009). This phenomenon is explained by the traction exerted by the invasive myofibroblasts that combine to realign collagen fibers into a ligament-like "strap" on the axis between the spheroids (Sawhney & Howard, 2002).

The collagen type I invasion assay makes use of transparent gels, is membrane-free, preserves cell morphology, allows for real-time monitoring, and has a kinetic flexibility. In contrast, in Transwell® chemo-invasion assays where cancer cells invade through a polycarbonate filter coated with a matrix substrate toward a chemoattractant, the number of cells crossing the filter can be counted at various time points. However, when invaded cells attach to the bottom of the polycarbonate filter the possibility to perform morphotypic and morphometric analysis is greatly compromised.

This protocol or parts of this protocol have been used successfully by us and others in the past (Vleminckx et al., 1991; Mooradian et al., 1992; Behrens et al., 1993; Barbier et al., 2001; De Wever et al., 2004a; b; Nystrom et al., 2005; Roperch et al., 2008; Hendrix et al., 2010).

8.4 Matrigel Invasion

A basement membrane (BM) aligns and anchors down epithelia to the underlying connective tissue and consequently forms a "barrier" for the passage of cells and macromolecules. Since BM degradation and invasion of cancer cells through the BM are key steps for determining malignancy of a tumor, a standardized, reproducible method was developed to study invasiveness of cancer cells. Currently, numerous variations based on this method allow us to study invasion not only with cancer cells, but with any cell type.

Matrigel is a reconstituted basement membrane extract that can be produced artificially in mice by inducing a BM-rich Engelbreth-Holm-Swarm (EHS) sarcoma from which basement membrane extracts containing laminin, collagen IV, heparan sulfate proteoglycan, nidogen, fibronectin and growth factors can be harvested (Grant et al., 1985). By using these extracts in a transwell system, proteolytic degradation of BM constituents and migration through this matrix can be studied (Albini et al., 1987; Albini & Benelli, 2007). In the conventional method, a standard transwell with a polycarbonate insert of 8 μm pore size (cat.no. 3422, Corning, Amsterdam, The Netherlands) is coated with Matrigel (Fig. 8.3). For inserts measuring 6.5 mm diameter, 20–60 μl Matrigel at a concentration of approximately 2–3 mg ml^{-1} mixture should be added on top of the membrane. Matrigel™ stock

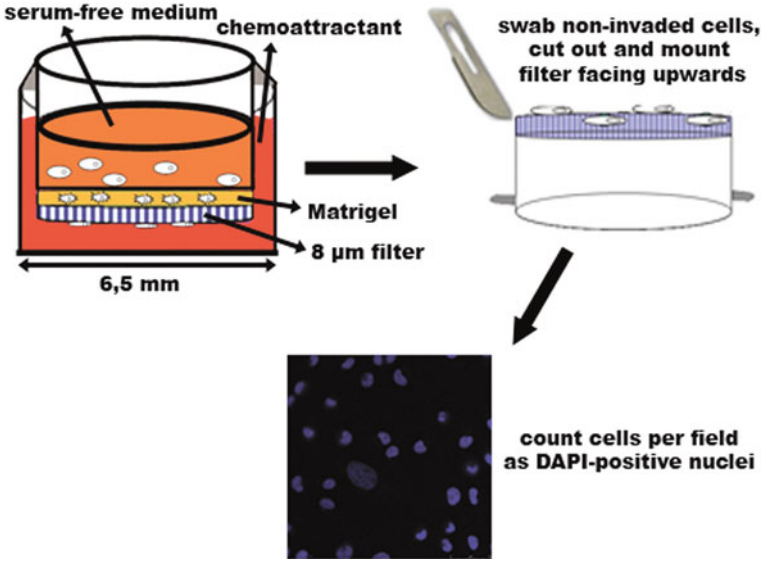

Fig. 8.3 Schematic representation of Matrigel invasion assay. Cells are seeded on top of the Matrigel-coated 8 µm pore size filter. Chemo-attractant, in serum-free conditions, is added to the lower compartment. After 24 h incubation, non-invaded cells on the upper side of the filter are swabbed and remaining, invasive cells are fixed in ice-cold methanol and DAPI stained. The filter is cut out of the insert and mounted in aqueous mounting medium with the bottom side facing upwards. Finally, numbers of DAPI-stained nuclei per field (50 fields) are counted using a fluorescence microscope

solutions as purchased from BD Biosciences (cat.no. 356234) or Sigma-Aldrich (cat.no. E1270) do not have absolute concentration indication, but in generally lies between 8 and 12 mg ml^{-1}. To obtain adequate working solution, the stock concentration should be diluted with serum-free culture media. After adding the diluted Matrigel on top of the filter, the 24-well plate needs to incubate at 37°C in humidified atmosphere with 10% CO_2 in air for at least 1 h. After gelification, cells have to be detached and collected by trypsinization to obtain a cell suspension of 5 to 50×10^4 cells ml^{-1} (depending on the cell type and duration of the assay), which has to be seeded on top the gelified Matrigel matrix. To attract the cells towards the bottom side of the porous membrane, a chemo-attractant must be applied in the lower compartment. Many factors can strongly affect chemo-invasion: the choice of the chemo-attractant is crucial, as single growth factors frequently show a weak activity on target cells, whereas more complex ''physiological'' attractants, such as conditioned media from other cell sources, can represent optimal ''cocktails'' active on several cell models. Naturally, the type and adequate concentration of chemo-attractant depends on the cell type used in the assay and should be established well before performing the experiment. Importantly, all the culture media used in the assay (Matrigel solution, chemo-attractant and cell suspension) need

to be serum-free to eliminate interference of serum proteins. Once the cells are seeded and the suitable chemo-attractant is applied in the lower compartment, the 24-well plate needs to incubate at 37°C in a humidified atmosphere and appropriate CO_2% for 24 h, although for certain cell types longer incubation periods can be applied. Next, the membrane's upper surface should be swabbed to remove non-invaded cells, the inserts are washed in Tris-Buffered Saline (TBS) solution and fixed in ice-cold methanol for 10 min. Nuclei are stained with DAPI (Sigma). Finally, invaded cells are counted in 50 fields/filter using a fluorescence microscope. Besides this classical counting method, fluorometric-based and real-time procedures are available (http://www.roche-applied-science.com/sis/xcelligence/ezhome.html).

As mentioned above, many variations on this conventional protocol exist to allow experiments with a variety of cell types or even co-culture systems to study cross-talk and the effect on invasion. The two main flexible parameters are the type of substratum and chemo-attractant used. Besides tumor or endothelial cells, mainly used in the conventional system, the Matrigel invasion assay is also used to study the invasive capacity of (myo)fibroblasts (De Wever et al., 2004b) in relation to cancer, rheumatoid arthritis (Tolboom & Huizinga, 2007; Sabeh et al., 2009a), and many other disorders. Thus in general, the main advantages of the Matrigel invasion assay are the flexibility of the assay, ease to experimental set-up and the low cost. On the other hand, disadvantages are the high variability of the results and the lack of morphological information during the experiment.

8.5 Monolayer Wound Healing Migration

The monolayer wound healing assay is based on the observation that, upon creation of a new artificial gap, so called "scratch", on a confluent cell monolayer, the cells on the edge of the newly created gap will migrate toward the opening to close the "scratch" until new cell-cell contacts are re-established. It is probably the most simple method to study cell migration in vitro and can be performed with the common and inexpensive supplies found in most cell culturing laboratories. Another major advantage of this simple method is that it mimics migration of cells in vivo to some extent. For example, the patterns of fibroblast migration as a loosely connected population mimic the behavior of these cells during migration in vivo. In addition monolayer wounding can also be used to examine vesicle trafficking involved in the establishment of cell polarity during directed locomotion.

Fibroblast cultures are seeded in one well of a 6-well culture dish. Before plating, two parallel lines are drawn at the bottom of the well with an ultra-fine tip marker. These lines will serve as reference points for the wound areas to be analyzed. At the day of analysis, the monolayer should be absolutely confluent (but not post-confluent). In preparation for making the wound, the complete growth medium is aspirated and replaced by calcium-free phosphate buffered saline (PBS) to prevent cell death at the edge of the wound by exposure to high calcium concentrations. Two parallel scratch wounds of approximately 400 μm width are made perpendicular

to the marker lines with a P100–200 pipet tip. This procedure makes it possible to image the entire width of the wound using a 10× objective. The wounds are observed using phase contrast microscopy on an inverted microscope. Images are taken at time 0 h (just after making the wound) and at regular intervals over the course of 12–24 h of both areas flanking the intersections of the wound and the marker lines. The width of the wound should be as consistent as possible, since narrow wounds tend to close faster than wider wounds. Images are analyzed by digitally drawing lines (using Adobe Photoshop) averaging the position of the migrating cells at the wound edges. The cell migration distance is determined by measuring the width of the wound divided by two and by subtracting this value from the initial half-width of the wound (Valster et al., 2005; Liang et al., 2007). Alternatively, confluent monolayers are wounded with a sterilized razor blade to remove part of the cell monolayer sheet (Andre et al., 1999) and assessed 24 h later by counting the number of intersections with marks at various distances from the original wound margin. Starting point of migration is revealed by a small incision in the culture dish that is made with the razor blade.

(Myo)fibroblasts move perpendicularly to the wound, in an irregular shaped front and not as a compact front like epithelial cells do (Suriano et al., 2003). In a recent study, perturbation of N-cadherin pathfinding activity by a neutralizing antibody or by a 10-mer N-cadherin peptide or by siRNA reduced directional migration, filopodia formation, polarization and Golgi-complex reorientation during wound healing of primary myofibroblasts (De Wever et al., 2004b). Furthermore, cell polarity is characterized by (1) a perpendicular orientation of the protrusion and the cell migration to the wound, and (2) a reorientation of the Golgi complex towards direction of migration. Another advantage of the wound healing assay is its particular suitability to study the regulation of cell migration by cell interaction with ECM. Here, plates are precoated with fibronectin, collagen type I or other ECM proteins. To produce a cell-free "scratch", 1-mm-thick steel plates can be inserted into wells before seeding the cells and should be removed again after the cells are attached to the bottom and become confluent. This method has the advantage over the scratching by pipet tip so that the substrate in the window is not destroyed.

Wound healing "scratch" assays may have a person-related variability and are not suitable for high-throughput screening. The Oris^{TM} Cell Migration Assay (Platypus Technologies LLC, Madison, Wi) (http://www.platypustech.com) is an alternative to the "scratch" assay. The Oris^{TM} Cell Migration Assay uses a multistep process that starts with the attachment of a detection mask to the bottom of the wells of the Oris^{TM} 96-well assay microplate. A specially designed, cell seeding stopper fitted inside of each microplate well restricts cells from seeding into a central detection zone as cells are being dispensed. After cell attachment has occurred (4–28 h), the stoppers are removed, allowing cells to freely migrate into the detection zone of the wells. Cells can be labeled pre- or post-migration. Cells that have not migrated into the detection zone are blocked from view by the mask initially applied to the plate bottom. Migrated cells may then be analyzed using a fluorescence plate reader.

8.6 Conclusion

In order to translate the findings from basic cellular research into clinical applications, cell-based models need to recapitulate both the three-dimensional organization and multicellular complexity of an organ but at the same time accommodate systematic experimental intervention. These models are feasible for studying a role for regulated exocytosis in migration/invasion. In addition, these systems allow (1) analysis of pro-/anti-invasive/migratory compounds, (2) analysis of gene dosage/knock down on invasion/migration, (3) identification of specific mechanisms that underlie invasion/migration.

References

Albini, A., & Benelli, R. (2007). The chemoinvasion assay: A method to assess tumor and endothelial cell invasion and its modulation. *Nature Protocols*, *2*, 504–511.

Albini, A., Iwamoto, Y., Kleinman, H. K., Martin, G. R., Aaronson, S. A., Kozlowski, J. M., et al. (1987). A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Research*, *47*, 3239–3245.

Andre, F., Rigot, V., Thimonier, J., Montixi, C., Parat, F., Pommier, G., et al. (1999). Integrins and E-cadherin cooperate with IGF-I to induce migration of epithelial colonic cells. *International Journal of Cancer*, *83*, 497–505.

Barbier, M., Attoub, S., Calvez, R., Laffargue, M., Jarry, A., Mareel, M., et al. (2001). Tumour biology. Weakening link to colorectal cancer? *Nature*, *413*, 796.

Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M. M., et al. (1993). Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/beta-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *The Journal of Cell Biology*, *120*, 757–766.

Burgoyne, R. D., & Morgan, A. (2003). Secretory granule exocytosis. *Physiological Reviews*, *83*, 581–632.

Chang, H. Y., Chi, J. T., Dudoit, S., Bondre, C., van de Rijn, M., Botstein, D., et al. (2002). Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*, *99*, 12877–12882.

De Wever, O., Demetter, P., Mareel, M., & Bracke, M. (2008). Stromal myofibroblasts are drivers of invasive cancer growth. *International Journal of Cancer*, *123*, 2229–2238.

De Wever, O., Hendrix, A., De Boeck, A., Westbroek, W., Braems, G., Emami, S., et al. (2009). Modeling and quantification of cancer cell invasion through collagen type I matrices. *International Journal of Developmental Biology*, doi: 10.1387/ijdb.092948ow.

De Wever, O., Nguyen, Q. D., Van Hoorde, L., Bracke, M., Bruyneel, E., Gespach, C., et al. (2004a). Tenascin-C and SF/HGF produced by myofibroblasts in vitro provide convergent pro-invasive signals to human colon cancer cells through RhoA and Rac. *The FASEB Journal*, *18*, 1016–1018.

De Wever, O., Westbroek, W., Verloes, A., Bloemen, N., Bracke, M., Gespach, C., et al. (2004b). Critical role of N-cadherin in myofibroblast invasion and migration in vitro stimulated by colon-cancer-cell-derived TGF-beta or wounding. *Journal of Cell Science*, *117*, 4691–4703.

Denys, H., Braems, G., Lambein, K., Pauwels, P., Hendrix, A., De Boeck, A., et al. (2009). The extracellular matrix regulates cancer progression and therapy response: Implications for prognosis and treatment. *Current Pharmaceutical Design*, *15*, 1373–1384.

Eyden, B. (2005). The myofibroblast: A study of normal, reactive and neoplastic tissues, with an emphasis on ultrastructure. Part 1 – Normal and reactive cells. *Journal of Submicroscopic Cytology and Pathology*, *37*, 109–204.

Friedrich, J., Seidel, C., Ebner, R., & Kunz-Schughart, L. A. (2009). Spheroid-based drug screen: Considerations and practical approach. *Nature Protocols*, *4*, 309–324.

Gailus-Durner, V., Fuchs, H., Becker, L., Brielmeier, M., Calzada-Wack, J., Elvert, R., et al. (2005). Introducing the German Mouse Clinic: Open access platform for standardized phenotyping. *Nature Methods*, *2*, 403–404.

Geppert, M., Goda, Y., Stevens, C. F., & Sudhof, T. C. (1997). The small GTP-binding protein Rab3A regulates a late step in synaptic vesicle fusion. *Nature*, *387*, 810–814.

Grant, D. S., Kleinman, H. K., Leblond, C. P., et al. (1985). The basement-membrane-like matrix of the mouse EHS tumor: II. Immunohistochemical quantitation of six of its components. *American Journal of Anatomy*, *174*, 387–398.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, *100*, 57–70.

Hendrix, A., Maynard, D., Pauwels, P., Braems, G., Denys, H., Van den Broecke, R., et al. (2010). The secretory small GTPase Rab27B regulates invasive tumor growth and metastasis through extracellular HSP90 alpha. *Journal of the National Cancer Institute*, In press.

Hentzer, B., & Kobayasi, T. (1978). Enzymatic liberation of viable cells of human skin. *Acta Dermato-Venereoogica*, *58*, 197–202.

Huizing, M., Helip-Wooley, A., Westbroek, W., Gunay-Aygun, M., & Gahl, W. A. (2008). Disorders of lysosome-related organelle biogenesis: Clinical and molecular genetics. *Annual Review Genomics and Human Genetics*, *9*, 359–386.

Jaiswal, J. K., Andrews, N. W., & Simon, S. M. (2002). Membrane proximal lysosomes are the major vesicles responsible for calcium-dependent exocytosis in nonsecretory cells. *Journal of Cell Biology*, *159*, 625–635.

Jensen, P. K., & Therkelsen, A. J. (1981). Cultivation at low temperature as a measure to prevent contamination with fibroblasts in epithelial cultures from human skin. *The Journal of Investigative Dermatology*, *77*, 210–212.

Jiao, X., Katiyar, S., Liu, M., Mueller, S. C., Lisanti, M. P., Li, A., et al. (2008). Disruption of c-Jun reduces cellular migration and invasion through inhibition of c-Src and hyperactivation of ROCK II kinase. *Molecular Biology of the Cell*, *19*, 1378–1390.

Liang, C. C., Park, A. Y., & Guan, J. L. (2007). In vitro scratch assay: A convenient and inexpensive method for analysis of cell migration in vitro. *Nature Protocols*, *2*, 329–333.

Matsushita, O., & Okabe, A. (2001). Clostridial hydrolytic enzymes degrading extracellular components. *Toxicon*, *39*, 1769–1780.

Mizuno, K., Tolmachova, T., Ushakov, D. S., Romao, M., Abrink, M., Ferenczi, M. A., et al. (2007). Rab27b regulates mast cell granule dynamics and secretion. *Traffic*, *8*, 883–892.

Mooradian, D. L., McCarthy, J. B., Komanduri, K. V., & Furcht, L. T. (1992). Effects of transforming growth factor-beta 1 on human pulmonary adenocarcinoma cell adhesion, motility, and invasion in vitro. *Journal of the National Cancer Institute*, *84*, 523–527.

Normand, J., & Karasek, M. A. (1995). A method for the isolation and serial propagation of keratinocytes, endothelial cells, and fibroblasts from a single punch biopsy of human skin. *In Vitro Cellular & Developmental Biology – Animal*, *31*, 447–455.

Nystrom, M. L., Thomas, G. J., Stone, M., Mackenzie, I. C., Hart, I. R., & Marshall, J. F. (2005). Development of a quantitative method to analyse tumour cell invasion in organotypic culture. *The Journal of Pathology*, *205*, 468–475.

Pellinen, T., Tuomi, S., Arjonen, A., Wolf, M., Edgren, H., Meyer, H., et al. (2008). Integrin trafficking regulated by Rab21 is necessary for cytokinesis. *Developmental Cell*, *15*, 371–385.

Ramalho, J. S., Anders, R., Jaissle, G. B., Seeliger, M. W., Huxley, C., & Seabra, M. C (2002). Rapid degradation of dominant-negative Rab27 proteins in vivo precludes their use in transgenic mouse models. *BMC Cell Biology*, *3*, 26.

Roperch, J. P., El Ouadrani, K., Hendrix, A., Emami, S., De Wever, O., Melino, G., et al. (2008). Netrin-1 induces apoptosis in human cervical tumor cells via the TAp73alpha tumor suppressor. *Cancer Research*, *68*, 8231–8239.

Sabeh, F., Li, X. Y., Saunders, T. L., Rowe, R. G., & Weiss, S. J. (2009a). Secreted versus membrane-anchored collagenases: Relative roles in fibroblast-dependent collagenolysis and invasion. *The Journal of Biological Chemistry*, *284*, 23001–23011.

Sabeh, F., Shimizu-Hirota, R., & Weiss, S. J. (2009b). Protease-dependent versus – independent cancer cell invasion programs: Three-dimensional amoeboid movement revisited. *Journal of Cell Biology*, *185*, 11–19.

Sawhney, R. K., & Howard, J. (2002). Slow local movements of collagen fibers by fibroblasts drive the rapid global self-organization of collagen gels. *Journal of Cell Biology*, *157*, 1083–1091.

Schmoranzer, J., Kreitzer, G., & Simon, S. M. (2003). Migrating fibroblasts perform polarized, microtubule-dependent exocytosis towards the leading edge. *Journal of Cell Science*, *116*, 4513–4519.

Shiflett, S. L., Kaplan, J., & Ward, D. M. (2002). Chediak-Higashi Syndrome: A rare disorder of lysosomes and lysosome related organelles. *Pigment Cell Research*, *15*, 251–257.

Sugimoto, H., Mundel, T. M., Kieran, M. W., Mateus, A. R., Ferreira, P., Casares, F., et al. (2006). Identification of fibroblast heterogeneity in the tumor microenvironment. *Cancer Biology & Therapy*, *5*, 1640–1646.

Suriano, G., Oliveira, M. J., Huntsman, D., et al. (2003). E-cadherin germline missense mutations and cell phenotype: Evidence for the independence of cell invasion on the motile capabilities of the cells. *Human Molecular Genetics*, *12*, 3007–3016.

Tolboom, T. C., & Huizinga, T. W. (2007). In vitro matrigel fibroblast invasion assay. *Methods in Molecular Medicine*, *135*, 413–421.

Valster, A., Tran, N. L., Nakada, M., Berens, M. E., Chan, A. Y., & Symons, M. (2005). Cell migration and invasion assays. *Methods*, *37*, 208–215.

van den Bogaerdt, A. J., van Zuijlen, P. P., van Galen, M., Lamme, E. N., & Middelkoop, E. (2002). The suitability of cells from different tissues for use in tissue-engineered skin substitutes. *Archives of Dermatological Research*, *294*, 135–142.

Vleminckx, K., Vakaet, L., Jr., Mareel, M., Fiers, W., & van Roy, F. (1991). Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell*, *66*, 107–119.

Wang, H., Van Blitterswijk, C. A., Bertrand-De Haas, M., Schuurman, A. H., & Lamme, E. N. (2004). Improved enzymatic isolation of fibroblasts for the creation of autologous skin substitutes. *In Vitro Cellular & Developmental Biology – Animal*, *40*, 268–277.

Ward, D. M., Griffiths, G. M., Stinchcombe, J. C., & Kaplan, J. (2000). Analysis of the lysosomal storage disease Chediak-Higashi syndrome. *Traffic*, *1*, 816–822.

Wels, J., Kaplan, R. N., Rafii, S., & Lyden, D. (2008). Migratory neighbors and distant invaders: Tumor-associated niche cells. *Genes & Development*, *22*, 559–574.

Wixler, V., Hirner, S., Muller, J. M., Gullotti, L., Will, C., Kirfel, J., et al. (2007). Deficiency in the LIM-only protein Fhl2 impairs skin wound healing. *Journal of Cell Biology*, *177*, 163–172.

Wu, M., Melichian, D. S., de la Garza, M., Gruner, K., Bhattacharyya, S., Barr, L., et al. (2009). Essential roles for early growth response transcription factor Egr-1 in tissue fibrosis and wound healing. *The American Journal of Pathology*, *175*, 1041–1055.

Chapter 9
Systemic Instigation: A Mouse Model to Study Breast Cancer as a Systemic Disease

Sandra S. McAllister

Abstract Little is known about the mechanisms that cause indolent tumors – such as micrometastases, occult primary tumors, or minimal residual disease – to erupt into overt, malignant cancers. As a result, predicting which patients are likely to experience disease relapse and treating patients with metastatic disease has been frustratingly limited. We developed an *in vivo* xenograft model system that provided us with fundamental insights into processes that govern indolent tumor growth and represented a new paradigm for translational cancer research. We learned that systemic endocrine factors and circulating bone marrow-derived cells support the acquisition of malignant traits by otherwise indolent tumors. As a result, we now think of cancer as a systemic disease by which tumors actively perturb as well as respond to the host systemic environment. First, we found that certain human breast carcinoma cell lines (we term "instigators") facilitate the growth of otherwise-indolent tumor cells (we term "responders") located at distant anatomical sites within host mice – a process we term "systemic instigation". Second, systemic instigation is accompanied by incorporation of bone marrow-derived cells into the stroma of the distant once-indolent tumors. Importantly, bone marrow cells (BMCs) of hosts bearing instigating tumors are functionally activated in the marrow prior to their mobilization into the circulation. Third, instigating tumor-derived osteopontin (OPN), a cytokine that is elevated in the plasma of patients with metastatic cancers and is predicitive of poor outcome, is necessary but not sufficient for systemic instigation. Although there may be alternative explanations, this systemic communication between tumors might explain why patients diagnosed with one malignant neoplasm are at an increased risk of presenting with multiple, independent primary cancers or why patients with recurrent disease often present with multiple metastases that appear to arise suddenly and synchronously. In this review, I address the methods by which the systemic instigation model was established, what we've learned by using this model, the implications of our studies, and some of the questions that have yet to be answered.

S.S. McAllister (✉)
Harvard Medical School, Boston, MA, USA; Hematology, Brigham and Women's Hospital, Boston, MA, USA
e-mail: smcallister1@partners.org

C. Brakebusch, T. Pihlajaniemi (eds.), *Mouse as a Model Organism*,

DOI 10.1007/978-94-007-0750-4_9,

9.1 The Clinical Challenges

Over the past few decades, the world has seen an unfortunate increase in the age-adjusted incidence of breast cancer. In the United States, one of every eight women is at risk of developing breast cancer and an estimated 40,000 women died of breast cancer in 2009 (Jemal et al., 2009). Nearly all cancer deaths are due to metastatic disease, even though tumor metastasis is considered to be an inefficient process. In some cases, tumor cells disseminate from primary sites at an early stage in tumor formation but remain indolent for protracted periods of time before becoming clinically detectable tumors (Nguyen, Bos, & Massague, 2009). The mechamisms by which these once-indolent tumors convert into vigorously growing cancers are not understood.

The view that tumor cells escape detection and remain indolent for protracted periods is not limited to disseminated, metastatic tumor cells but can also apply to primary tumors or minimal residual disease. In fact, indolent tumors are thought to be prevalent in the general population and upon autopsy, people who died of causes other than cancer were found to have some type of indolent tumor or carcinoma in situ (Black & Welch, 1993; Folkman & Kalluri, 2004; Nielsen, Thomsen, Primdahl, Dyreborg, & Andersen, 1987).

The period of time during which tumors exhibit no net change in size has been referred to as "dormancy". Although tumor dormancy has been defined primarily in the experimental setting (Almog et al., 2006; Gohongi et al., 1999; Kang & Watnick, 2008; Naumov et al., 2006; O'Reilly et al., 1994), clinical observations support the concept of dormancy (Aguirre-Ghiso, 2007; Fehm et al., 2008). Nevertheless, the mechanisms governing tumor dormancy and those dictating whether indolent tumors will emerge into overt, malignant tumors are not clear. One theory, which has been intensively investigated, is that the efficiency with which a tumor's blood supply is formed will dictate whether the tumor will maintain or escape from dormancy (reviewed in Almog, 2010). As a result, the angiogenic process has become an attractive target for therapy; however, anti-angiogenic therapies have met with only limited success and in some cases, have accelerated disease recurrence and aggressiveness (Casanovas, Hicklin, Bergers, & Hanahan, 2005; Ebos, Lee, & Kerbel, 2009; Paez-Ribes et al., 2009).

Recent technical advances allow one to characterize disseminated and circulating tumor cells (DTCs and CTCs, respectively) that are isolated from the blood or bone marrow of cancer patients. Researchers have isolated single CTCs from the blood of cancer patients at early stages in the disease state (Nagrath et al., 2007) while others have shown that DTCs are present in the bone marrow even during the early hyperplastic stage of tumor formation (Klein, 2009; Pantel, Cote, & Fodstad, 1999). Gene expression profiling of metastatic tumors and analysis of DTCs from breast cancer patients also support the notion that cells are disseminated early in tumor development and might evolve independently of and in parallel with the primary tumor (Klein et al., 2002; Ramaswamy, Ross, Lander, & Golub, 2003; Schmidt-Kittler et al., 2003; van 't Veer et al., 2002; van de Vijver et al., 2002; Vecchi et al., 2008; Woelfle et al., 2003). Despite the ability to detect these single cells, it is currently

impossible to test whether they are endowed with the ability to form overt tumors; likewise, it is impossible to predict whether these CTCs/DTCs are indeed the cells that would cause a patient to become symptomatic.

As a result of the challenges enumerated above, predicting which patients are likely to experience disease relapse and treating patients with metastatic disease has been frustratingly limited. It has become increasingly clear that more relevant preclinical models are necessary in order to enhance our understanding of factors that contribute to the malignant growth of otherwise indolent tumor cells. The development of such models will most certainly serve as a prerequisite for designing more effective diagnostic tests and therapies to treat cancer patients.

9.2 The Discovery of Systemic Instigation

In our efforts to understand how indolent tumors are stimulated to become frank carcinoma, we made the surprising discovery that an aggressively growing breast tumor (we termed "instigator") promoted the growth of an otherwise indolent breast tumor (we termed "responder") located at a distant anatomical site without itself metastasizing to that site (McAllister et al., 2008). In other words, malignant growth of the responding tumor was governed by systemic endocrine cues delivered by the distant instigating tumor. We termed this action-at-a-distance, "systemic instigation".

Our findings were evocative of earlier reports demonstrating that multiple tumors within a mouse host could affect one another from a distance. In some experimental mouse isograft studies, anti-angiogenic factors secreted by a subcutaneous tumor inhibited the outgrowth of lung metastases (Gohongi et al., 1999; O'Reilly et al., 1997; 1994; Volpert, Lawler, & Bouck, 1998). In other studies, multiple tumor burden enhanced the growth of otherwise latent cancers (Mullen, Urban, Van Waes, Rowley, & Schreiber, 1985); in these studies, the presence of an immune-suppressor tumor growing in one anatomical site enabled the progression of otherwise-weakly tumorigenic foci at distant sites. More recent use of experimental models has revealed that certain tumors orchestrate a variety of events that take place in anatomical locations that are distant from where such tumors reside (for review, see McAllister & Weinberg, 2010).

As a result of these advances and our discovery of systemic instigation, we think of cancer as a disease that is capable of perturbing as well as responding to the host systemic environment. The most obvious extrapolation of such observations is the metastatic setting in which a primary tumor supports the growth of its metastases; however, the implications of the systemic instigation model are not limited to such a scenario. In cancer patients, tumor burden also exists in the form of multiple primary tumor foci (e.g. contralateral breast cancer), minimal residual disease, and multiple metastases that arise after a primary tumor has been removed. Thus, we classify instigators as any type of tumor that is capable of impacting the host systemic environment in such a way as to support the growth of otherwise indolent or poorly growing tumors that are capable of responding to such systemic stimuli.

9.3 The Systemic Instigation Cascade

Systemic instigation is a complex, multi-step process. We currently have a broad understanding of these biological processes and are only beginning to understand some of the molecular and cellular mediators involved in the systemic instigation cascade as follows:

9.3.1 Instigating Tumors Facilitate Growth of Responding Tumors at Distant Anatomical Locations

The most obvious and relevant consequence of the systemic instigation cascade is the growth of tumors that would otherwise remain indolent. We initially established the systemic instigation model by injecting aggressively growing BPLER breast tumor cells subcutaneously into one flank of immunocompromised Nude mice and indolent HMLER-HR responding breast cancer cells into the contralateral flanks of these mice (Fig. 9.1a). BPLER cells are transformed primary mammary epithelial cells that form aggressively growing tumors following implantation into immunocompromised mice (Ince et al., 2007). Upon histopathological analysis, the resulting BPLER tumors look much like human adenocarcinomas encountered in

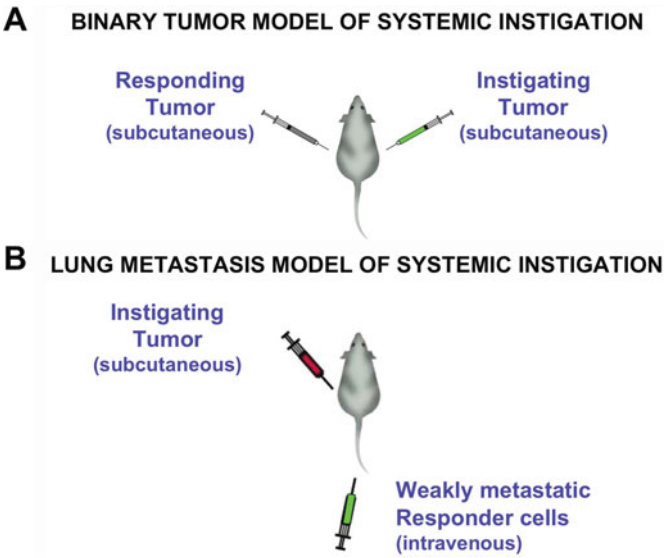

Fig. 9.1 *Injection schemes for systemic instigation experiments.* **a** Aggressively growing instigating tumors are injected subcutaneously into one flank and indolent responder cells are injected subcutaneously into the contralateral flank of host Nude mice. **b** Instigating tumors are injected subcutaneously into one flank and weakly metastatic cells are injected into the tail vein of host Nude mice

the oncology clinic (Ince et al., 2007; McAllister et al., 2008). The responding HMLER-HR cells are also transformed mammary epithelial cells; however, these cells are poorly tumorigenic when injected into immunocompromised mice and in most cases, remain indolent for protracted periods of time (for as long as 16 weeks following injection) (Elenbaas et al., 2001; McAllister et al., 2008).

As we reported, when we injected the indolent HMLER-HR cells (responders) opposite the BPLER cells (instigators) in Nude mice, both the average mass of the responding tumors and the incidence of responding tumor formation were increased as compared to those responders injected opposite control Matrigel vehicle control (McAllister et al., 2008). In an alternative protocol to this model, we injected the instigating cells into one flank of the Nude mice and allowed them to grow for a period of 30 days before injecting the responding tumors cells. In this instance, the responding tumors started to form palpable masses within the first 2 weeks of their injection, without requiring the 40-day lag period that resulted when the two cell types had been injected simultaneously (McAllister et al., 2008). This finding revealed that the instigating systemic environment, once established, is powerful enough to stimulate a rapid response.

In addition to the experimentally transformed BPLER instigating tumor cell line, we identified and reported a number of other instigating breast cancer cell lines (McAllister et al., 2008). Importantly, we also reported the discovery of a non-instigating tumor cell line (PC3 prostate carcinoma) that, despite its own vigorous growth, was unable to instigate distant responding tumor growth. Subsequent to our initial findings, we identified a non-instigating breast cancer cell line, BT474, which was unable to facilitate growth of the contralaterally implanted breast carcinoma responder cells (unpublished observation).

9.3.2 Instigating Tumors Promote Metastatic Colonization

It is thought that in order to form successful metastases, epithelial tumor cells must execute a number of distinct processes including: local invasion into the surrounding tissue, intravasation into the lymphatic and/or hematogenous circulatory systems, survival in the circulation, lodging and extravasation into the parenchyma of distant organs, survival in the distant organs as single-celled micrometastases, and out-growth, or “colonization”, into macroscopic tumors (Fidler, 2003). Colonization is thought to be a rate-limiting step in cancer progression during which time micrometastatic foci must overcome impediments to their growth in order to form overt tumors (Chambers, Naumov, Vantyghem, & Tuck, 2000; Paget, 1889).

Traditional models of metastasis suggested that small subclones of cells that are endowed with the ability to metastasize are rare within primary tumors and emerge late in tumor progression (Fidler & Kripke, 1977). These ideas have been challenged by more recent clinical and experimental evidence demonstrating that metastatic cells are disseminated early in primary tumor formation (Bernards & Weinberg, 2002). In some patients, metastases are diagnosed in the absence of primary tumor detection, and epidemiological studies concluded that metastases had initiated prior

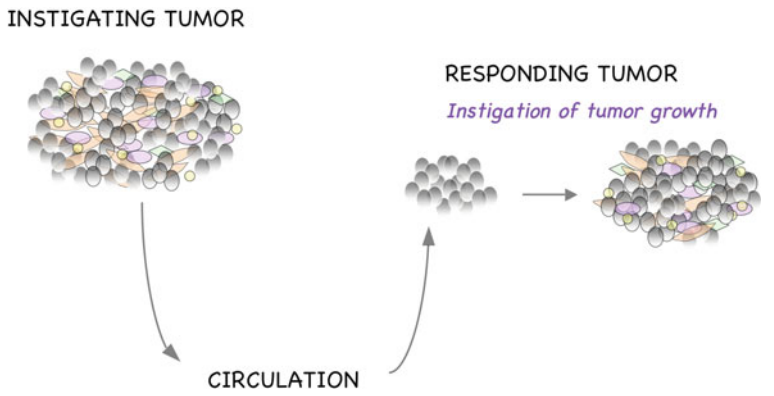

Fig. 9.2 Instigating tumors promote the growth of responding tumors in a systemic endocrine fashion

to diagnosis of primary tumors (Ansieau, Hinkal, Thomas, Bastid, & Puisieux, 2008; Engel et al., 2003). The fact that disease can recur years after the primary tumor is removed suggests that these disseminated cells remain indolent for protracted periods of time. In fact, it would seem that the dissemination of tumor cells from a primary site occurs at a very high frequency, since autopsy of cancer patients nearly always reveals the presence of indolent metastases (Weiss, 1992).

As reported, when we intravenously injected weakly metastatic cells into the tail veins of Nude mice, we found that the presence of a subcutaneous instigating tumor enhanced their ability to colonize the lungs (McAllister et al., 2008) (Fig. 9.1b). The instigating tumor did not affect the initial arrival of cells to the lungs, nor did it impact the clearance of the majority of the cells from the lungs that typically takes place during the first 48 h after intravenous injection of tumor cells. Rather, the instigating tumor promoted colonization of the otherwise weakly metastatic cells that had successfully disseminated to the lungs. The number of visible metastatic foci in the lungs of mice bearing subcutaneous instigating tumors was significantly elevated above those of mice bearing subcutaneous non-instigating tumors (McAllister et al., 2008).

Therefore, we view instigation as a process that is independent of the sites where indolent responder cells reside (Fig. 9.2). Moreover, we learned that even small instigating tumors were endowed with these instigating powers, as tumors that represented only 0.04% of total body mass were capable of facilitating responding tumor growth (McAllister et al., 2008). Taken together, these findings support the notion that systemic instigation might operate between: (i) primary tumors and their metastases; (ii) different primary tumors; or, (iii) independent metastatic foci.

9.3.3 Instigating Tumors Secrete Osteopontin

We reported the discovery of a molecular mediator that is necessary for systemic instigation – tumor-derived osteopontin (OPN). We found that, in general, instigating human xenografts secrete OPN while non-instigators do not (McAllister

et al., 2008). When analyzed relative to the indolent HMLER-HR breast tumor cells, OPN expression (as measured via QPCR) and secretion of OPN protein into the mouse plasma (as measured by ELISA) were significantly elevated in the instigating tumors.

Osteopontin (also known as secreted phosphoprotein 1, spp1, and early T cell activation gene 1, Eta-1) was originally discovered as a secreted phosphoprotein product of transformation (Senger, Wirth, & Hynes, 1979) and as a bone matrix protein (Franzen & Heinegard, 1985). Subsequently, an extensive body of research has demonstrated that OPN expression extends to a variety of tissues and fluids and its function as a cytokine spans a variety of normal and pathological processes including bone remodeling, inflammation, mammary gland morphogenesis, wound healing, atherosclerosis, and tumor metastasis (Cho & Kim, 2009; Cook et al., 2005; Denhardt, Noda, O'Regan, Pavlin, & Berman, 2001; Nemir et al., 2000). OPN is detected in the blood of disease-free men and women, and in women, the plasma OPN levels are not affected by menstrual cycle or menopause (Furger, Menon, Tuck, Bramwell, & Chambers, 2001).

OPN is not typically mutated to confer pathology; rather, it is subject to extensive post-translational modifications (PTMs), including O-glycosylation, serine/threonine phosphorylation, and proteolytic cleavage (Kazanecki, Uzwiak, & Denhardt, 2007b). These PTMs occur in a cell- and tissue-specific manner and generate OPN protein products with differing biological activities that are well conserved across species. The most studied of these activities include cell adhesion, migration, and survival via its interactions with integrins and CD44. Currently, post-translational modifications of tumor-derived OPN have not been fully characterized, although it is speculated that tumor-derived OPN is significantly less phosphorylated than bone forms of OPN (Christensen et al., 2007; Kazanecki, Kowalski, Ding, Rittling, & Denhardt, 2007a). Experimental evidence confirmed that tumor derived OPN is soluble and not matrix associated (Rittling, Chen, Feng, & Wu, 2002) and that the activity of tumor-derived OPN is functionally different from that of OPN secreted by macrophages (Crawford, Matrisian & Liaw, 1998). Currently, we do not know the form of OPN that is endowed with "instigating" activity.

The relevance of OPN expression to human cancer pathogenesis has been revealed by studies showing that OPN expression levels are elevated in aggressive tumors types when compared with normal tissue or low-grade tumors, correlates with the presence of metastatic disease, and is included among lists of genes that predict poor prognosis in patients with various types of cancer (Likui, Hong, & Shuwen, 2009; Minn et al., 2005; Richardson et al., 2006; van de Vijver et al., 2002). A number of other studies have shown that soluble OPN is detected at elevated levels in the blood of cancer patients with metastatic disease and is also predictive of poor prognosis (Ramankulov et al., 2007; Rudland et al., 2002; Tuck, Chambers, & Allan, 2007).

In our studies, OPN was necessary for systemic instigation. When we suppressed OPN secretion from instigating tumors through the use of short hairpin RNAs against OPN, their instigating ability was abolished (McAllister et al., 2008). Interestingly, although these tumors lost their instigating power, their own growth was unaffected. Currently, we do not know why OPN is dispensable for instigating tumors; however, our results are consistent with other experimental models of

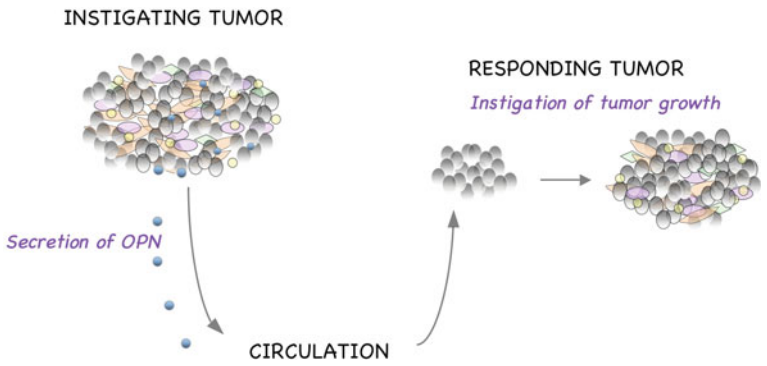

Fig. 9.3 Instigating tumors secrete osteopontin (OPN), which is necessary but not sufficient for systemic instigation of responding tumor growth

metastasis that revealed that OPN deficiency did not affect the growth of certain primary tumors, yet it significantly reduced their metastatic potential (Cook et al., 2005; Feng & Rittling, 2000; Wai et al., 2005). In these earlier reports, the ability of OPN to enhance cell motility and invasion *in vitro* was presented as one explanation for the role of OPN in enhancing metastasis. We identified an alternative, novel role for tumor-derived OPN in supporting distant tumor growth that could only be revealed *in vivo* – OPN affects these distant tumors in an indirect systemic fashion (Johnston & El-Tanani, 2008) (Fig. 9.3).

OPN alone is not sufficient for systemic instigation; hence, when we ectopically expressed OPN in non-instigating tumors, they failed to facilitate contralateral responding tumor formation *in vivo* (McAllister et al., 2008). Therefore, we conclude that additional, unidentified factors cooperate with OPN to mediate the systemic instigation cascade.

9.3.4 Bone Marrow Cells Serve as the Intermediaries Between Instigating and Responding Tumors

We reported that bone marrow cells were incorporated into responding tumors to a significantly greater extent when they were implanted contralaterally to instigating tumors than when implanted opposite non-instigating tumors (McAllister et al., 2008). To perform these experiments, we harvested bone marrow cells (BMCs) from immunocompromised mice that ubiquitously express green fluorescent protein ($Rag1^{-/-}$EGFPTg mice) and transplanted them into irradiated Nude mice. We developed these $Rag1^{-/-}$EGFPTg donor mice, which are devoid of functional T cells and B cells, in order to avoid potential complications associated with graft-versus-graft or graft-versus-host disease when human xenografts were subsequently implanted into the immunocompromised Nude mice following bone marrow transplantation.

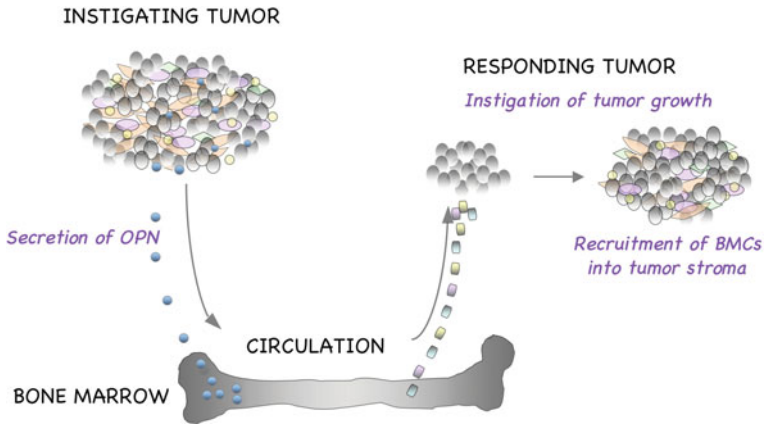

Fig. 9.4 Bone marrow cells are recruited into the stroma of responding tumor only in the presence of a distant instigating tumor

In our experiments, the non-instigators recruited at least as many bone marrow derived cells as the instigators. Therefore, we concluded that only instigating tumors are capable of providing the distant responding tumors with the stromal cells that they require for growth. Our finding that BMCs are directed to responding tumors by distant instigating tumors represented an important advance beyond previously published literature, which predominantly addressed bone marrow cell recruitment to primary tumors. Recruitment of bone marrow-derived cells into responding tumor stroma thus serves as a hallmark of the systemic instigation cascade (Fig. 9.4).

There are a number of experimental and clinical studies to suggest that some tumor stromal cells have origins in the bone marrow. Some reports demonstrated that some female cancer patients who received bone marrow allografts from male donors had cells in their tumor stroma that were of donor origin (Direkze et al., 2004; Worthley et al., 2009). Intense research efforts have been focused on bone marrow-derived cells that are recruited into various tumors due to their tumor-promoting paracrine effects (Joyce & Pollard, 2009; Kopp, Ramos, & Rafii, 2006; Murdoch, Muthana, Coffelt, & Lewis, 2008). Certainly, the inflammatory and immune cells that are prevalent in some carcinomas have origins in the bone marrow (Coussens & Werb, 2002). At present, a detailed analysis of the precise types of bone marrow cells that are recruited into the responding tumors in the instigating environment is under investigation.

9.3.5 Instigating Tumors Activate Bone Marrow Cells Prior to Their Mobilization

We ascribed the process of systemic instigation, in great part, to the ability of instigating tumors to perturb the host bone marrow. While bone marrow cells were

incorporated into the responding tumors, as described above, we needed a way to determine whether these BMCs were playing a role in the instigation process. Thus, we devised a test of bone marrow function. By harvesting BMCs from the marrows of various tumor-bearing mice, mixing these BMCs directly with responder cells, and implanting these mixtures subcutaneously into Nude mice, we could determine whether the BMCs were functionally activated to support tumor growth (McAllister et al., 2008).

By employing this test, we found and reported that BMCs of hosts bearing instigating tumors were functionally activated even prior to their mobilization into the circulation; hence, when co-injected with responder cells, the activated BMCs mimicked the effects imparted by instigating tumors. Bone marrow cells from non-instigating or control mice did not have this activity. Thus, the activated bone marrow is unique to instigator-bearing hosts.

Various tissues have been found to release endocrine signals under certain conditions, such as wound healing, that are transmitted to the bone marrow, where these signals mobilize multipotent stem cells and induce them or their progeny to enter the circulation (Badiavas, Abedi, Butmarc, Falanga, & Quesenberry, 2003; Fathke et al., 2004). Elevated levels of such circulating endocrine factors have also been correlated with tumor progression and metastasis in cancer patients (Holzer et al., 2001; Poon et al., 2001; Ugurel, Rappl, Tilgen, & Reinhold, 2001). For instance, some tumors release pro-angiogenic factors, which induce mobilization of hematopoietic and endothelial precursor cells into the circulation from the bone marrow to support angiogenesis (Heissig et al., 2002; Moore et al., 2001; Rafii, 2000).

Our work demonstrated that BMCs are activated even prior to their mobilization and that this activation occurs in an OPN-dependent fashion (Fig. 9.5). We

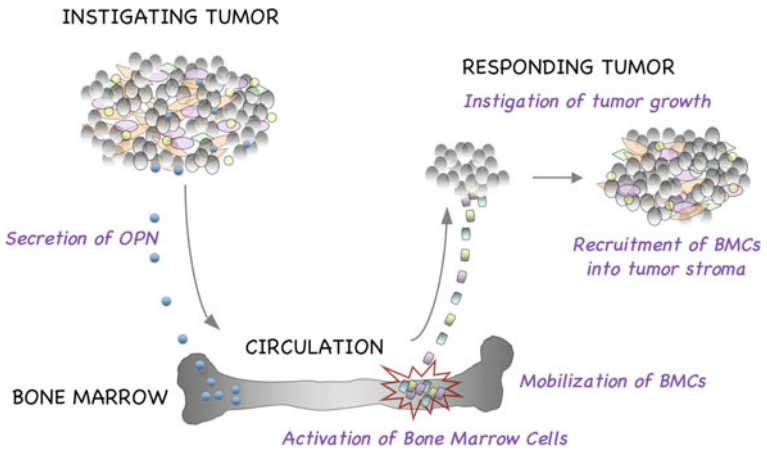

Fig. 9.5 Bone marrow cells of instigating tumor-bearing hosts are functionally activated, in an OPN-dependent manner, to support responding tumor growth even prior to their mobilization into the host circulation

reported that in the absence of OPN (expressing shRNA against OPN in the instigating tumors), the BMCs of the instigator-bearing hosts were no longer able to support tumor growth (McAllister et al., 2008). Together with other reports (Shojaei et al., 2007), our results support the theory that bone marrow activation and mobilization are two separate steps of the instigation cascade; however, we do not yet have experimental evidence that these events are indeed uncoupled.

9.3.6 Instigating Tumors Perturb Primitive Hematopoietic Cells in the Bone Marrow

As published in our earlier report, we consistently observed a subtle, yet significant decrease in the Lin-/Sca1+/cKit+ (LSK) primitive hematopoietic compartment of the bone marrow in mice bearing instigating tumors as compared with that of control animals (McAllister et al., 2008). Mice bearing non-instigating tumors did not display this depression of the LSK population, and the presence of the LSK population in their marrow was no different from that of control animals.

We learned that instigating tumor-derived osteopontin was necessary for this suppression of the LSK compartment. Other studies first alerted us to the possibility that OPN might mediate at least part of the systemic instigation mechanism. These studies demonstrated that OPN signaling regulates the fate of primitive hematopoietic cells *in vitro* and, perhaps more importantly, that OPN-deficient mice have significantly elevated levels of LSK cells in their bone marrow (Iwata, Awaya, Graf, Kahl, & Torok-Storb, 2004; Nilsson et al., 2005; Stier et al., 2005). Under the converse situation, when instigating tumor-derived OPN was elevated in the plasma of host mice, the LSK cells were less frequent in the bone marrow of instigator-bearing mice than in that of control animals (McAllister et al., 2008).

Therefore, suppression of the LSK bone marrow population is indicative of both the presence of an instigating tumor and elevated levels of functional OPN (Fig. 9.6). Nevertheless, at present, we do not know whether the LSK suppression is related to the functional activation of cells in the marrow of animals bearing instigating tumors.

9.4 The Mice

Our current systemic instigation protocols rely on the use of immunocompromised mice as hosts for human tumor xenografts. We have learned that the selection of immunocompromised mice strain used for systemic instigation studies is an important determinant. We made the provocative observation that systemic instigation, as measured by responding tumor growth, is robust in Nude mice while it is significantly less efficient in other immunocompromised strains, including NOD-SCID and Rag1$^{-/-}$ mice (unpublished observations). Paradoxically, Nude mice are the least immunocompromised of these strains. In fact, Nude mice have B cells, T cell

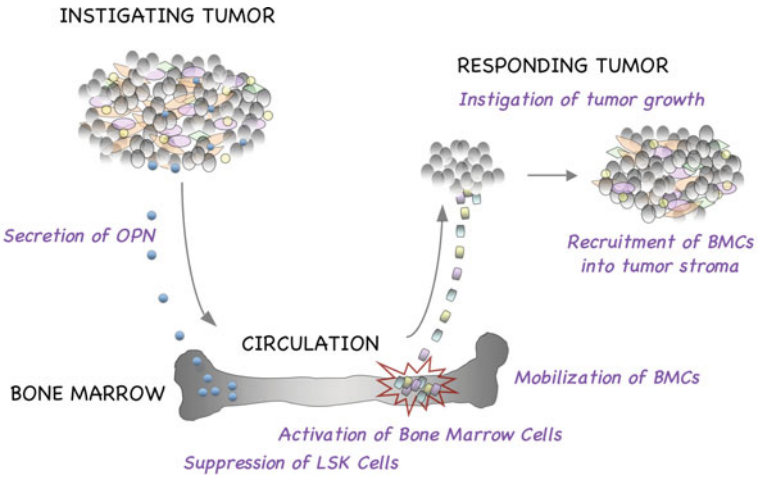

Fig. 9.6 *The systemic instigation cascade.* Instigating tumors secrete osteopontin (OPN), resulting in reduced numbers of primitive hematopoietic cells (LSK) in the host bone marrow; cells in the bone marrow of hosts bearing instigating tumors are functionally activated prior to mobilization into the circulation; activated bone marrow-derived cells are released into the circulation and subsequently incorporated into distant responding tumor stroma. The consequence of this cascade of systemic events is the malignant growth of once-indolent responder cells

precursors in the bone marrow, NK cells, and a full complement of cells of the innate immune system (Pritchard & Micklem, 1973; Schedi, Goldstein, & Boyce, 1975; Takigawa & Hanaoka, 1978; Welsh, 1978; Wortis, 1971).

It is possible therefore, that our findings indicate a tumor-supportive role of some component of the immune system that is operational in Nude mice but not in other immunocompromised strains of mice. While there are alternative explanations for these discrepancies, published experimental evidence points to a tumor-supportive role of some immune cells, including B cells (Andreu et al., 2010; de Visser, Korets, & Coussens, 2005), which are functional in the Nude mice. As a result of these findings, the Nude mouse is the mouse of choice for systemic instigation studies using human tumor xenografts.

9.5 The Experimental Applications

In order to understand the powers of systemic instigation, we tested whether human tumor surgical specimens could be induced to grow when implanted contralaterally to instigating tumors in host mice. Most attempts to grow human surgical specimens as xenografts in immunocompromised mice are met with a low success rate (Peehl, 2004; Visonneau, Cesano, Torosian, Miller, & Santoli, 1998).

By applying the systemic instigation model to real human tumor samples, we reported that fragments of a human colon tumor, which otherwise did not grow

in host mice, grew vigorously in the presence of contralateral instigating tumors (McAllister et al., 2008). These findings indicated the generality of this physiologic signaling and revealed that our *in vivo* model system, or derivatives thereof, might be used to study the growth of human tumor specimens that might otherwise grow very slowly or not at all as xenografts. Because the histopathology of instigated tumor specimens was similar to that of the freshly resected primary tumor specimens from cancer patients, and because implant success rate and growth rate were both significantly increased, we reason that our *in vivo* systemic instigation model might prove useful for studying real human tumor biology in the laboratory setting.

9.6 The Implications of Systemic Instigation

Our results indicate that cancer is a systemic disease long before primary tumors become metastatic. Thus, we have documented perturbation of the bone marrow in tumor-bearing hosts when the instigating tumor is less than 0.08% of total body mass. Our results identify a novel role for OPN that does not contradict previously described roles of OPN in metastasis (Johnston & El-Tanani, 2008). They do, in addition, help to explain the repeatedly observed elevated concentrations of OPN in the circulation of patients suffering advanced metastatic disease.

Previous paradigms of metastasis suggested that overt metastases could only arise from cells that are intrinsically endowed with the ability to grow in a foreign environment at metastatic sites or through their acquisition of additional genetic mutations. Our work revealed a somewhat surprising shift in this paradigm – metastatic colonization can be governed on a systemic level by endocrine factors and bone marrow-derived cells whose activities are orchestrated by tumors at distant sites.

It is possible that the systemic interactions between distinct tumors that coexist in a given host reflect phenomena observed in the oncolgy clinic. It has been reported that patients diagnosed with one malignant neoplasm are at an increased risk of presenting with multiple, independent primary cancers within a relatively short time period after the initial diagnosis (Schoenberg, 1977; Watanabe et al., 1984). As an example, some breast cancer patients develop contralateral breast cancer, which is a predictor of poor outcome (Schaapveld et al., 2008a, b), and patients with synchronous bilateral breast cancer tend to have a significantly worse overall survival than those patients with metachronous or unilateral cancers (Carmichael, Bendall, Lockerbie, Prescott, & Bates, 2002). In some cases, patients with recurrent disease present with multiple metastases that appear to arise suddenly and synchronously. Moreover, as was recently reported, surgical resection of primary breast cancers significantly improved the survival time of patients who presented with distant metastases at the time of their primary diagnosis (Ruiterkamp et al., 2009; Ruiterkamp et al., 2010). While there are a number of possible explanations for these diverse clinical observations, we propose that understanding the systemic cross-talk between tumors might provide some insight into these phenomena. Perhaps as we acquire a deeper understanding of the mechanisms that provoke

the malignant growth of tumors that would otherwise remain indolent, we can design better individual therapies for cancer patients.

Acknowledgments I thank Dr. Robert A. Weinberg and members of his laboratory for intellectual contributions and critical discussions during the design and implementation of the experiments reviewed herein. Additionally, I thank Ann M. Gifford and Hanna Kuznetsov for technical support and contributions toward unpublished observations mentioned in this review. Finally, I wish to acknowledge Drs. Zafira Castaño Corsino and Moshe Elkabets for helpful discussions.

References

Aguirre-Ghiso, J. A. (2007). Models, mechanisms and clinical evidence for cancer dormancy. *Nature Reviews Cancer*, *7*, 834–846.

Almog, N. (2010). Molecular mechanisms underlying tumor dormancy. *Cancer Letters*, *294*, 139–146.

Almog, N., Henke, V., Flores, L., Hlatky, L., Kung, A. L., Wright, R. D., et al. (2006). Prolonged dormancy of human liposarcoma is associated with impaired tumor angiogenesis. *The FASEB Journal*, *20*, 947–949.

Andreu, P., Johansson, M., Affara, N. I., Pucci, F., Tan, T., Junankar, S., et al. (2010). FcRgamma activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell*, *17*, 121–134.

Ansieau, S., Hinkal, G., Thomas, C., Bastid, J., & Puisieux, A. (2008). Early origin of cancer metastases: Dissemination and evolution of premalignant cells. *Cell Cycle*, *7*, 3659–3663.

Badiavas, E. V., Abedi, M., Butmarc, J., Falanga, V., & Quesenberry, P. (2003). Participation of bone marrow derived cells in cutaneous wound healing. *Journal of Cellular Physiology*, *196*, 245–250.

Bernards, R., & Weinberg, R. A. (2002). A progression puzzle. *Nature*, *418*, 823.

Black, W. C., & Welch, H. G. (1993). Advances in diagnostic imaging and overestimations of disease prevalence and the benefits of therapy. *New England Journal of Medicine*, *328*, 1237–1243.

Carmichael, A. R., Bendall, S., Lockerbie, L., Prescott, R., & Bates, T. (2002). The long-term outcome of synchronous bilateral breast cancer is worse than metachronous or unilateral tumours. *European Journal of Surgical Oncology*, *28*, 388–391.

Casanovas, O., Hicklin, D. J., Bergers, G., & Hanahan, D. (2005). Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell*, *8*, 299–309.

Chambers, A. F., Naumov, G. N., Vantyghem, S. A., & Tuck, A. B. (2000). Molecular biology of breast cancer metastasis. Clinical implications of experimental studies on metastatic inefficiency. *Breast Cancer Research*, *2*, 400–407.

Cho, H. J., & Kim, H. S. (2009). Osteopontin: A multifunctional protein at the crossroads of inflammation, atherosclerosis, and vascular calcification. *Current Atherosclerosis Reports*, *11*, 206–213.

Christensen, B., Kazanecki, C. C., Petersen, T. E., Rittling, S. R., Denhardt, D. T., & Sorensen, E. S. (2007). Cell type-specific post-translational modifications of mouse osteopontin are associated with different adhesive properties. *Journal of Biological Chemistry*, *282*, 19463–19472.

Cook, A. C., Tuck, A. B., McCarthy, S., Turner, J. G., Irby, R. B., Bloom, G. C., et al. (2005). Osteopontin induces multiple changes in gene expression that reflect the six "hallmarks of cancer" in a model of breast cancer progression. *Molecular Carcinogenesis*, *43*, 225–236.

Coussens, L. M., & Werb, Z. (2002). Inflammation and cancer. *Nature*, *420*, 860–867.

Crawford, H. C., Matrisian, L. M., & Liaw, L. (1998). Distinct roles of osteopontin in host defense activity and tumor survival during squamous cell carcinoma progression in vivo. *Cancer Research*, *58*, 5206–5215.

de Visser, K. E., Korets, L. V., & Coussens, L. M. (2005). De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell*, *7*, 411–423.

Denhardt, D. T., Noda, M., O'Regan, A. W., Pavlin, D., & Berman, J. S. (2001). Osteopontin as a means to cope with environmental insults: Regulation of inflammation, tissue remodeling, and cell survival. *The Journal of Clinical Investigation*, *107*, 1055–1061.

Direkze, N. C., Hodivala-Dilke, K., Jeffery, R., Hunt, T., Poulsom, R., Oukrif, D., et al. (2004). Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts. *Cancer Research*, *64*, 8492–8495.

Ebos, J. M., Lee, C. R., & Kerbel, R. S. (2009). Tumor and host-mediated pathways of resistance and disease progression in response to antiangiogenic therapy. *Clin Cancer Research*, *15*, 5020–5025.

Elenbaas, B., Spirio, L., Koerner, F., Fleming, M. D., Zimonjic, D. B., Donaher, J. L., et al. (2001). Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes & Development*, *15*, 50–65.

Engel, J., Eckel, R., Kerr, J., Schmidt, M., Furstenberger, G., Richter, R., et al. (2003). The process of metastasisation for breast cancer. *The European Journal of Cancer*, *39*, 1794–1806.

Fathke, C., Wilson, L., Hutter, J., Kapoor, V., Smith, A., Hocking, A., et al. (2004). Contribution of bone marrow-derived cells to skin: Collagen deposition and wound repair. *Stem Cells*, *22*, 812–822.

Fehm, T., Mueller, V., Marches, R., Klein, G., Gueckel, B., Neubauer, H., et al. (2008). Tumor cell dormancy: Implications for the biology and treatment of breast cancer. *APMIS*, *116*, 742–753.

Feng, F., & Rittling, S. R. (2000). Mammary tumor development in MMTV-c-myc/MMTV-v-Ha-ras transgenic mice is unaffected by osteopontin deficiency. *Breast Cancer Research and Treatment*, *63*, 71–79.

Fidler, I. J. (2003). The pathogenesis of cancer metastasis: The 'seed and soil' hypothesis revisited. *Nature Reviews Cancer*, *3*, 453–458.

Fidler, I. J., & Kripke, M. L. (1977). Metastasis results from preexisting variant cells within a malignant tumor. *Science*, *197*, 893–895.

Folkman, J., & Kalluri, R. (2004). Cancer without disease. *Nature*, *427*, 787.

Franzen, A., & Heinegard, D. (1985). Isolation and characterization of two sialoproteins present only in bone calcified matrix. *Biochemical Journal*, *232*, 715–724.

Furger, K. A., Menon, R. K., Tuck, A. B., Bramwell, V. H., & Chambers, A. F. (2001). The functional and clinical roles of osteopontin in cancer and metastasis. *Current Molecular Medicine*, *1*, 621–632.

Gohongi, T., Fukumura, D., Boucher, Y., Yun, C. O., Soff, G. A., Compton, C., et al. (1999). Tumor-host interactions in the gallbladder suppress distal angiogenesis and tumor growth: Involvement of transforming growth factor beta1. *Nature Medicine*, *5*, 1203–1208.

Heissig, B., Hattori, K., Dias, S., Friedrich, M., Ferris, B., Hackett, N. R., et al. (2002). Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell*, *109*, 625–637.

Holzer, G., Obermair, A., Koschat, M., Preyer, O., Kotz, R., & Trieb, K. (2001). Concentration of vascular endothelial growth factor (VEGF) in the serum of patients with malignant bone tumors. *Medical and Pediatric Oncology*, *36*, 601–604.

Ince, T. A., Richardson, A. L., Bell, G. W., Saitoh, M., Godar, S., Karnoub, A. E., et al. (2007). Transformation of different human breast epithelial cell types leads to distinct tumor phenotypes. *Cancer Cell*, *12*, 160–170.

Iwata, M., Awaya, N., Graf, L., Kahl, C., & Torok-Storb, B. (2004). Human marrow stromal cells activate monocytes to secrete osteopontin, which down-regulates Notch1 gene expression in CD34+ cells. *Blood*, *103*, 4496–4502.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., & Thun, M. J. (2009). Cancer statistics, 2009. *CA: A Cancer Journal for Clinicians*, *59*, 225–249.

Johnston, N. I., & El-Tanani, M. K. (2008). Osteopontin: A new role for a familiar actor. *Breast Cancer Research*, *10*, 306.

Joyce, J. A., & Pollard, J. W. (2009). Microenvironmental regulation of metastasis. *Nature Reviews Cancer*, *9*, 239–252.

Kang, S. Y., & Watnick, R. S. (2008). Regulation of tumor dormancy as a function of tumor-mediated paracrine regulation of stromal Tsp-1 and VEGF expression. *APMIS*, *116*, 638–647.

Kazanecki, C. C., Kowalski, A. J., Ding, T., Rittling, S. R., & Denhardt, D. T. (2007a). Characterization of anti-osteopontin monoclonal antibodies: Binding sensitivity to post-translational modifications. *Journal of Cellular Biochemistry*, *102*, 925–935.

Kazanecki, C. C., Uzwiak, D. J., & Denhardt, D. T. (2007b). Control of osteopontin signaling and function by post-translational phosphorylation and protein folding. *Journal of Cellular Biochemistry*, *102*, 912–924.

Klein, C. A. (2009). Parallel progression of primary tumours and metastases. *Nature Reviews Cancer*, *9*, 302–312.

Klein, C. A., Blankenstein, T. J., Schmidt-Kittler, O., Petronio, M., Polzer, B., Stoecklein, N. H., et al. (2002). Genetic heterogeneity of single disseminated tumour cells in minimal residual cancer. *Lancet*, *360*, 683–689.

Kopp, H. G., Ramos, C. A., & Rafii, S. (2006). Contribution of endothelial progenitors and proangiogenic hematopoietic cells to vascularization of tumor and ischemic tissue. *Current Opinion in Hematology*, *13*, 175–181.

Likui, W., Hong, W., & Shuwen, Z. (2010). Clinical significance of the upregulated osteopontin mRNA expression in human colorectal cancer, *The Journal of Gastrointestinal Surgery*, *14*, 74–81.

McAllister, S. S., Gifford, A. M., Greiner, A. L., Kelleher, S. P., Saelzler, M. P., Ince, T. A., et al. (2008). Systemic endocrine instigation of indolent tumor growth requires osteopontin. *Cell*, *133*, 994–1005.

McAllister, S. S., & Weinberg, R. A. (2010). Tumor-host interactions: A far-reaching relationship. *Journal of Clinical Oncology*, *28*, 4022–4028.

Minn, A. J., Gupta, G. P., Siegel, P. M., Bos, P. D., Shu, W., Giri, D. D., et al. (2005). Genes that mediate breast cancer metastasis to lung. *Nature*, *436*, 518–524.

Moore, M. A., Hattori, K., Heissig, B., Shieh, J. H., Dias, S., Crystal, R. G., et al. (2001). Mobilization of endothelial and hematopoietic stem and progenitor cells by adenovector-mediated elevation of serum levels of SDF-1, VEGF, and angiopoietin-1. *Annals of the New York Academy of Sciences*, *938*, 36–45, discussion 45–37.

Mullen, C. A., Urban, J. L., Van Waes, C., Rowley, D. A., & Schreiber, H. (1985). Multiple cancers. Tumor burden permits the outgrowth of other cancers. *Journal of Experimental Medicine*, *162*, 1665–1682.

Murdoch, C., Muthana, M., Coffelt, S. B., & Lewis, C. E. (2008). The role of myeloid cells in the promotion of tumour angiogenesis. *Nature Reviews Cancer*, *8*, 618–631.

Nagrath, S., Sequist, L. V., Maheswaran, S., Bell, D. W., Irimia, D., Ulkus, L., et al. (2007). Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature*, *450*, 1235–1239.

Naumov, G. N., Bender, E., Zurakowski, D., Kang, S. Y., Sampson, D., Flynn, E., et al. (2006). A model of human tumor dormancy: An angiogenic switch from the nonangiogenic phenotype. *Journal of the National Cancer Institute*, *98*, 316–325.

Nemir, M., Bhattacharyya, D., Li, X., Singh, K., Mukherjee, A. B., & Mukherjee, B. B. (2000). Targeted inhibition of osteopontin expression in the mammary gland causes abnormal morphogenesis and lactation deficiency. *The Journal of Biological Chemistry*, *275*, 969–976.

Nguyen, D. X., Bos, P. D., & Massague, J. (2009). Metastasis: From dissemination to organ-specific colonization. *Nature Reviews Cancer*, *9*, 274–284.

Nielsen, M., Thomsen, J. L., Primdahl, S., Dyreborg, U., & Andersen, J. A. (1987). Breast cancer and atypia among young and middle-aged women: A study of 110 medicolegal autopsies. *British Journal of Cancer*, *56*, 814–819.

Nilsson, S. K., Johnston, H. M., Whitty, G. A., Williams, B., Webb, R. J., Denhardt, D. T., et al. (2005). Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood*, *106*, 1232–1239.

O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., et al. (1997). Endostatin: An endogenous inhibitor of angiogenesis and tumor growth. *Cell*, *88*, 277–285.

O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., et al. (1994). Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell*, *79*, 315–328.

Paez-Ribes, M., Allen, E., Hudock, J., Takeda, T., Okuyama, H., Vinals, F., et al. (2009). Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell*, *15*, 220–231.

Paget, S. (1889). The distribution of secondary growths in cancer of the breast. *Lancet*, *1*, 571–573.

Pantel, K., Cote, R. J., & Fodstad, O. (1999). Detection and clinical importance of micrometastatic disease. *Journal of the National Cancer Institute*, *91*, 1113–1124.

Peehl, D. M. (2004). Are primary cultures realistic models of prostate cancer? *Journal of Cellular Biochemistry*, *91*, 185–195.

Poon, R. T., Ng, I. O., Lau, C., Zhu, L. X., Yu, W. C., Lo, C. M., et al. (2001). Serum vascular endothelial growth factor predicts venous invasion in hepatocellular carcinoma: A prospective study. *Annals of Surgery*, *233*, 227–235.

Pritchard, H., & Micklem, H. S. (1973). Haemopoietic stem cells and progenitors of functional T-lymphocytes in the bone marrow of 'nude' mice. *Clinical & Experimental Immunology*, *14*, 597–607.

Rafii, S. (2000). Circulating endothelial precursors: Mystery, reality, and promise. *The Journal of Clinical Investigation*, *105*, 17–19.

Ramankulov, A., Lein, M., Kristiansen, G., Meyer, H. A., Loening, S. A., & Jung, K. (2007). Elevated plasma osteopontin as marker for distant metastases and poor survival in patients with renal cell carcinoma. *Journal of Cancer Research and Clinical Oncology*, *133*, 643–652.

Ramaswamy, S., Ross, K. N., Lander, E. S., & Golub, T. R. (2003). A molecular signature of metastasis in primary solid tumors. *Nature Genetics*, *33*, 49–54.

Richardson, A. L., Wang, Z. C., De Nicolo, A., Lu, X., Brown, M., Miron, A., et al. (2006). X chromosomal abnormalities in basal-like human breast cancer. *Cancer Cell*, *9*, 121–132.

Rittling, S. R., Chen, Y., Feng, F., & Wu, Y. (2002). Tumor-derived osteopontin is soluble, not matrix associated. *Journal of Biological Chemistry*, *277*, 9175–9182.

Rudland, P. S., Platt-Higgins, A., El-Tanani, M., De Silva Rudland, S., Barraclough, R., Winstanley, J. H., et al. (2002). Prognostic significance of the metastasis-associated protein osteopontin in human breast cancer. *Cancer Research*, *62*, 3417–3427.

Ruiterkamp, J., Ernst, M. F., van de Poll-Franse, L. V., Bosscha, K., Tjan-Heijnen, V. C., & Voogd, A. C. (2009). Surgical resection of the primary tumour is associated with improved survival in patients with distant metastatic breast cancer at diagnosis, *European Journal of Surgical Oncology*, *35*, 1146–1151.

Ruiterkamp, J., Voogd, A. C., Bosscha, K., Tjan-Heijnen, V. C., & Ernst, M. F. (2010). Impact of breast surgery on survival in patients with distant metastases at initial presentation: A systematic review of the literature. *Breast Cancer Research and Treatment*, *120*, 9–16.

Schaapveld, M., Visser, O., Louwman, W. J., Willemse, P. H., de Vries, E. G., van der Graaf, W. T., et al. (2008b). The impact of adjuvant therapy on contralateral breast cancer risk and the prognostic significance of contralateral breast cancer: A population based study in the Netherlands. *Breast Cancer Research and Treatment*, *110*, 189–197.

Schaapveld, M., Visser, O., Louwman, M. J., de Vries, E. G., Willemse, P. H., Otter, R., et al. (2008a). Risk of new primary nonbreast cancers after breast cancer treatment: A Dutch population-based study. *Journal of Clinical Oncology*, *26*, 1239–1246.

Schedi, M. P., Goldstein, G., & Boyce, E. A. (1975). Differentiation of T cells in nude mice. *Science*, *190*, 1211–1213.

Schmidt-Kittler, O., Ragg, T., Daskalakis, A., Granzow, M., Ahr, A., Blankenstein, T. J., et al. (2003). From latent disseminated cells to overt metastasis: Genetic analysis of systemic breast cancer progression. *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 7737–7742.

Schoenberg, B. S. (1977). Multiple primary malignant neoplasms. The connecticut experience, 1935–1964. *Recent Results Cancer Research*, *58*, 1–173.

Senger, D. R., Wirth, D. F., & Hynes, R. O. (1979). Transformed mammalian cells secrete specific proteins and phosphoproteins. *Cell*, *16*, 885–893.

Shojaei, F., Wu, X., Malik, A. K., Zhong, C., Baldwin, M. E., Schanz, S., et al. (2007). Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. *Nature Biotechnology*, *25*, 911–920.

Stier, S., Ko, Y., Forkert, R., Lutz, C., Neuhaus, T., Grunewald, E., et al. (2005). Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *The Journal of Experimental Medicine*, *201*, 1781–1791.

Takigawa, M., & Hanaoka, M. (1978). In vivo maturation of B cells in the spleen of nude mice following administration of bacterial lipopolysaccharide. *International Archives of Allergy and Applied Immunology*, *56*, 115–122.

Tuck, A. B., Chambers, A. F., & Allan, A. L. (2007). Osteopontin overexpression in breast cancer: Knowledge gained and possible implications for clinical management. *Journal of Cellular Biochemistry*, *102*, 859–868.

Ugurel, S., Rappl, G., Tilgen, W., & Reinhold, U. (2001). Increased serum concentration of angiogenic factors in malignant melanoma patients correlates with tumor progression and survival. *Journal of Clinical Oncology*, *19*, 577–583.

van de Vijver, M. J., He, Y. D., van't Veer, L. J., Dai, H., Hart, A. A., Voskuil, D. W., et al. (2002). A gene-expression signature as a predictor of survival in breast cancer. *The New England Journal of Medicine*, *347*, 1999–2009.

van 't Veer, L. J., Dai, H., van de Vijver, M. J., He, Y. D., Hart, A. A., Mao, M., et al. (2002). Gene expression profiling predicts clinical outcome of breast cancer. *Nature*, *415*, 530–536.

Vecchi, M., Confalonieri, S., Nuciforo, P., Vigano, M. A., Capra, M., Bianchi, M., et al. (2008). Breast cancer metastases are molecularly distinct from their primary tumors. *Oncogene*, *27*, 2148–2158.

Visonneau, S., Cesano, A., Torosian, M. H., Miller, E. J., & Santoli, D. (1998). Growth characteristics and metastatic properties of human breast cancer xenografts in immunodeficient mice. *The American Journal of Pathology*, *152*, 1299–1311.

Volpert, O. V., Lawler, J., & Bouck, N. P. (1998). A human fibrosarcoma inhibits systemic angiogenesis and the growth of experimental metastases via thrombospondin-1. *Proceedings of the National Academy of Sciences of the United States of America*, *95*, 6343–6348.

Wai, P. Y., Mi, Z., Guo, H., Sarraf-Yazdi, S., Gao, C., Wei, J., et al. (2005). Osteopontin silencing by small interfering RNA suppresses in vitro and in vivo CT26 murine colon adenocarcinoma metastasis. *Carcinogenesis*, *26*, 741–751.

Watanabe, S., Kodama, T., Shimosato, Y., Arimoto, H., Sugimura, T., Suemasu, K., et al. (1984). Multiple primary cancers in 5,456 autopsy cases in the National Cancer Center of Japan. *Journal of the National Cancer Institute*, *72*, 1021–1027.

Weiss, L. (1992). Comments on hematogenous metastatic patterns in humans as revealed by autopsy. *Clinical and Experimental Metastasis*, *10*, 191–199.

Welsh, R. M., Jr. (1978). Mouse natural killer cells: Induction specificity, and function. *The Journal of Immunology*, *121*, 1631–1635.

Woelfle, U., Cloos, J., Sauter, G., Riethdorf, L., Janicke, F., van Diest, P., et al. (2003). Molecular signature associated with bone marrow micrometastasis in human breast cancer. *Cancer Research*, *63*, 5679–5684.

Worthley, D. L., Ruszkiewicz, A., Davies, R., Moore, S., Nivison-Smith, I., Bik To, L., et al. (2009). Human gastrointestinal neoplasia-associated myofibroblasts can develop from bone marrow-derived cells following allogeneic stem cell transplantation. *Stem Cells*, *27*, 1463–1468.

Wortis, H. H. (1971). Immunological responses of 'nude' mice. *Clinical & Experimental Immunology*, *8*, 305–317.

Index

C. Brakebusch, T. Pihlajaniemi (eds.), *Mouse as a Model Organism*,
DOI 10.1007/978-94-007-0750-4,