Chapter 2
Basic Concepts of Reproductive Biology and Genetics

2.1 Introduction

This chapter brings together a variety of information and concepts that are important for understanding the following chapters. The first section is an overview concerning mouse reproductive biology and embryology. This topic is important because, nowadays, many experiments in genetics require the manipulation of embryos at different stages of development, either to study their phenotype or for the production of chimeras with other embryos or with genetically engineered embryonic stem (ES) cells. The second part is a compilation of concepts of general or molecular genetics related to the phenotypic expression of mutations. More information can also be retrieved from several websites, where books and manuals are freely available online.[1]

2.2 Reproduction in the Laboratory Mouse

2.2.1 The Estrous Cycle and Pregnancy

Laboratory mice are *polyestrous* mammals. This means that, provided they are raised and housed in a suitable environment, the animals can reproduce all year round with only a small decline in fertility during the winter season.[2] In females, sexual maturity (puberty) takes place gradually from the age of 3–4 weeks. The vaginal orifice, which is normally sealed at birth by an epithelial operculum, opens

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[1] The website http://informatics.jax.org/ is a fundamental database resource for the laboratory mouse, providing integrated genetic, genomic, and biological data. It is a true "gold mine" for mouse geneticists to which we will frequently refer. Several books dealing with some fundamental aspects of mouse biology are freely available at this website.

[2] The reproductive activity of wild mice is interrupted or reduced during winter. This period is called *anestrus*.

between 25 and 40 days. From 6 to 8 weeks after birth, and depending on the strain, ovulation starts, and, in principle, all females older than 8 weeks are able to reproduce, exhibiting a typical cyclic sexual activity. Male puberty occurs slightly earlier, sometimes as early as 5 weeks, usually at 6–8 weeks.

The female reproductive cycle, the *estrous cycle*, lasts 4–6 days and is arbitrarily divided into four stages with the following order: *proestrus*, *estrus*, *metestrus*, and *diestrus*.[3] Proestrus and metestrus last about one day each, while the estrous period lasts only 12–16 h. Diestrus is the last and longest stage of the estrous cycle (~2 days).

Based on vaginal cytology, embryologists have defined criteria that characterize the four stages of the mouse estrous cycle (Byers et al. 2012). According to these criteria the estrus period is characterized by the presence of many flat and keratinized epithelial cells that are obvious upon examination of vaginal swabs. These cells are eosinophilic, meaning that they are stained deep red by the dye eosin. These visible changes during the estrous cycle reflect the variations in progesterone and estrogen levels. Female mice copulate only during the estrous period, which is often designated the "*heat period*" by analogy with the sexual behavior of other domestic females. The heat period lasts about 12 h and mating generally occurs during the first half of the night. In mice, matings are uncommon during the day.[4]

By using the above-described cytological parameters it is possible to identify and sort out the female mice that are in the estrous phase of the cycle, and, accordingly, that are hormonally prepared to copulate. However, this procedure is tedious and labor-intensive, especially when many females are to be selected, and for this reason it is not used very much. In practice, researchers prefer to select the female mice that are in the best conditions to mate by examining the external vaginal morphology (Byers et al. 2012). In this case, the vulva is slightly swelled and the vagina is slightly open. This kind of selection requires some experience but it is fast, quite reliable, and has the enormous advantage of not stressing the mice in a critical period.

The proestrus and estrus phases of the cycle are often designated the *follicular phase* because it is at the end of this phase that a batch of mature oocytes is released from the ovarian follicles. This generally occurs during or immediately after copulation, but copulation is not a prerequisite for this to occur because mice are spontaneous ovulators. If males are not present in the cage, ovulation will still normally occur during estrus.

Shortly after copulation, the fluids secreted by the various sexual glands of the males (in particular the seminal vesicles and the coagulating glands), which are components of the male's ejaculate, coagulate to form a vaginal plug. The plug in

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[3] *Estrus*, sometimes spelled *oestrus* (UK), is a noun; *estrous* (*oestrous*) is the corresponding adjective.

[4] For some precisely timed pregnancies, female mice must sometimes be bred in a "light-reversed" environment.

question tightly seals the vaginal lumen and prevents any further mating.[5] The vaginal plug is a relatively hard substance and remains in the female's vagina for several hours (up to 6–8 h or even more). During this time the vaginal plug progressively resorbs and the spermatozoa are released. Detection of a vaginal plug means that mating occurred during the preceding hours, but does not guarantee that pregnancy will ensue.[6]

By analogy with the follicular phase, metestrus and diestrus constitute the *luteal phase*. During this phase the *corpus luteum* forms and replaces the follicle. The *corpora lutea* secrete the hormone progesterone, the hormone of pregnancy, and persist until the end of pregnancy—if pregnancy ensues. If not, the corpora lutea degenerate and a new cycle starts. Corpora lutea are easy to recognize at the surface of the mouse ovary because they are slightly protuberant and often stained light orange. After fixation with formalin or Bouin's fixative, their identification is even easier.

When virgin or non-pregnant females are housed in groups and mated with males without prior selection of the phase of the estrous cycle, the frequency of natural mating is not evenly distributed over the following nights. On the contrary, one generally observes a peak after the third night of mating, indicating that some synchronization of the estrous cycle occurred. Synchronization of the estrous cycle by the presence of a male has been reported and is called the *Whitten effect* (Whitten 1956). It is a consequence of the dispersion in the environment of volatile pheromones that are at high concentration in the urine of males; these pheromones interfere with the hormonal control of the female cycle.

Fertilization of the oocytes takes place 10–15 h after ovulation, in the upper segment of the female reproductive tract, more precisely during their transit through the Fallopian tubes or oviducts (sometimes called *ampulla*). When the head of a sperm cell succeeds in penetrating the oocyte after passing through the *zona pellucida* (also designated *oolemma*), the penetration of other sperm cells is blocked and this triggers the completion of the second meiotic division. The second polar body from the oocyte is ejected within two hours; the male pronucleus expands, and finally the two haploid pronuclei (male and female) fuse, and the oocyte becomes an egg (i.e., a diploid embryo that is not yet implanted). Segmentation in the embryo begins slowly at first. 68–72 h after fertilization (i.e., at the beginning of the 4th day after mating), the embryos enter the uterus and implant into the uterine wall at the late or expanded blastocyst stage.

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[5] Such a vaginal plug is specific to the *Mus* genus and does not exist, for example, in the rat. Whether it confers a selective advantage to the species is an open question.

[6] As mentioned, most matings occur during the night; this is why "plugging" must be achieved preferably during the morning of the following day. Detection of a plug is sometimes very easy, especially when it bulges out of the vagina. In other instances, a probe may be necessary to detect resistance when gently inserted into the vagina. The type of probe used by ophthalmologists to unclog the tear ducts of human patients is a perfect tool for this task.

Embryologists date the different stages of pregnancy from the day the vaginal plug is discovered—i.e., day E0.5 by convention.[7]

Starting at 12–14 days of gestation, it is possible to detect the fetuses implanted inside the uterus, which feel like "rosary beads" to the touch. To do this, the female must be held firmly by the skin of its neck and back, with its abdomen overturned, and gently palpated with the fingers of the other hand once the abdominal wall is relaxed. Around 12 days of gestation, the pregnant females start to gain weight and will soon show abdominal bulging; this can be another way to confirm pregnancy by comparison with age-matched non-pregnant females.

Matching the number of corpora lutea with the actual number of fetuses implanted in the uterine horns allows one to compute the number of conceptuses that were possibly lost before implantation. This may be important, for example, when an embryonic lethal mutation is suspected to be responsible for the reduction in the size of the progeny. In normal conditions, the number of corpora lutea, which can be counted directly under a magnifying glass corresponds to the number of implanted fetuses (see Sect. 2.2.7 on twinning).

The gestation period ranges from 19 to 22 days but this depends upon a number of parameters. For example, females that are pregnant for the first time (primiparous) deliver their progeny up to 1 day before multiparous females of the same strain. The duration of pregnancy also varies slightly from one strain to another. For example, pregnancy is, on the average, 1 day longer in mice of strain DBA/2 than in mice of strain C57BL/6.

At the end of the gestation period, the corpora lutea degenerate (*luteolysis*), inducing parturition.[8] The pelvic girdle of the females relaxes and parturition begins in the following 2–4 h.[9] During the same period, the behavior of the female changes dramatically. The female is hyperactive and appears to have only one thing in mind: preparing a nest in a corner of the cage, preferably in a darker area.

Parturition generally occurs at night and may last up to 3 h, depending on the litter size. The fetuses are expelled one after the other, giving the mother time to take care of each of the pups. The fetal membrane and the placenta, as well as the dead embryos, if any, are carefully removed and ingested by the mother.[10] Embryos are also stimulated for breathing by repeated gentle pressure of the mother's paws on the thorax of the newborns. Once the last pup has been delivered and carefully revived, the mother lays over all the newborns gathered in the nest and lactation starts. Newborn mice are hairless, deaf, and blind, and are unable to regulate their body temperature

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[7] Dating the different steps of mouse embryonic development has been a matter of controversy. Some embryologists wanted the first day of pregnancy to be designated day 1; others argued that it should be day 0. In fact, the most accurate dating takes into account that, when the vaginal plug is discovered, the embryo is at 0.5 days of development. At this time it is a one-cell embryo just after fertilization (E0.5) (based on Theiler 1972).

[8] Resorption of the corpora lutea is triggered by prostaglandins secreted by the placenta.

[9] A gentle pressure on the pelvis of the mouse allows one to detect the relaxation of the pelvic girdle.

[10] Making the observation of non-viable (stillbirth) phenotypes difficult.

for the first 2 days of life *ab utero*; this is why the mother leaves the nest for only brief periods, only to feed, defecate, and drink. Lactation normally lasts 3–4 weeks depending on the number and degree of vigor of the pups. In the mouse, the number of neonates is frequently greater than the number of nipples (10), but this is not a problem and the pups are generally fed adequately.[11] From the age of 12–14 days, the young mice start eating solid food and the mother's milk is only a complement to the diet. At the end of the lactation period, in general at the end of the third week of life, the young mice are weaned and separated according to their sex by the technicians.

The standard reproductive cycle we have just described is sometimes modified to fit with practical contingences. For example, adoption and foster nursing are common practices in laboratory mouse breeding colonies, especially when the number of progeny is low or the mother is not particularly good at nursing. When there are only one or two pups in a progeny, the mother frequently abandons it/them, presumably because the stimulation of milk production is insufficient. If this situation occurs, it is then wise to take no risk and to transfer the secluded pups as early as possible into an age-matched (up to 1 day younger) litter.[12] Mice dams, unlike many other female mammals, generally accept adopted pups to nurse and milk, especially when they are young. Newborns selected for adoption can be simply added or exchanged in equal numbers with pups of the foster mother. It is recommended, when possible, that the newborns to be adopted be put in contact with some urine-soaked wood-shavings taken from the mother's bedding prior to the transfer, to expose them to the foster mother's smell.

Female mice can deliver up to eight progenies in their sexual life, depending on the strain. However, the progeny size decreases after the fourth progeny and, most importantly, the time that elapses between two successive progenies increases after the third progeny. The number of progeny one can expect from a group of female breeders can be evaluated based on the breeding records.[13] Males can breed for a very long time, sometimes up to 2 years; however, they are normally replaced after 10–12 months, depending on the strain.

Although mice are legendary for having exceptional aptitudes with regard to reproduction in the wild, the situation is different in laboratory conditions and sometimes requires special care. Reproduction and sexual behavior can be influenced by a number of parameters that are not always easy to control. Pheromones, for example, which are true olfactory hormones, play a major role in this matter. The mouse is probably more affected by pheromones than any other mammal, because of the complexity of its olfactory functions. Pheromones are proteins which are released into the urine, the skin secretions, and the saliva of males and

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[11] If this is not the case, the pups are left outside of the nest; they progressively cool, do not move much, and have no milk in their stomachs. Foster nursing is then urgent.

[12] Selecting a mother nursing a litter with a different coat color (albino/non-albino) is a clever way to check the success of the adoption without perturbing the mother.

[13] A useful and reliable criterion is the average number of mice weaned per mated female per week.

which modify the behavior of females. We have already reported the *Whitten effect* (synchronization of estrous cycle) that affects female mice when they are housed in groups. In addition to this observation, when females are kept in the absence of male pheromones (which is not easy to achieve in practice), this leads to a state of anestrus (lack of a normal estrus cycle). This phenomenon is called the *Lee– Boot effect* (Van der Lee and Boot 1956). Finally, it is sometimes observed that females, although found with a vaginal plug, never get pregnant when housed in close vicinity with some males. This phenomenon is known as the *Bruce effect* and an explanation is that the pheromones of the males prevent embryo implantation. The males in question are called "*strange males*" (Bruce 1959).

Nutrition is another major parameter that must be seriously taken into account concerning mouse reproduction. Since laboratory animals are fed exclusively on industrial (pelleted) diets, it is extremely important to make sure that the diet constantly provides the optimal amount of nutrients and vitamins, even after sterilization by heat or gamma rays. Some vitamins (C, B1, B9 for example) are extremely heat-sensitive but yet are essential to the function of reproduction; it is therefore essential to frequently change the heat-sterilized food. Nutritional deficiencies are difficult to diagnose but they are insidious and almost always have consequences on fertility, even if the mice do not exhibit any other obvious signs.

Environmental conditions (temperature, ventilation, noise, light cycle) are other parameters to be controlled with care. Noise and vibrations are probably the worst, especially when discontinuous, because the animals cannot become familiar with them and are in constant stress. When the airflow bothers the animals they generally protect themselves and their nest by building a bulwark with their bedding. This is a good indication that something is wrong with the air-conditioning system or the airflow inside the individually ventilated cage. Environmental enrichment like nesting materials and igloos are highly recommended to improve the breeding performance of a mouse colony.

Finally, infectious diseases are also extremely important and must be carefully monitored. Some viruses that cause unapparent diseases have a strong influence on fertility, either because they interfere with the production gametes or because they result in abortions or stillbirths. For more details concerning husbandry and maintenance of laboratory mice, consult the books by Fox et al. (2007) and Hedrich (2012).

2.2.2 Inducing Ovulation in the Mouse (Superovulation)

The information provided in the preceding section concerning mouse reproduction will be useful for those scientists who are willing to run a breeding unit. However, in many cases, geneticists are only interested in harvesting large quantities of fertilized eggs, for example, for creating transgenic animals by pronuclear injection, or for making chimeras, or simply for the preservation of embryos at low temperature. In some other cases, researchers are only willing to collect unfertilized oocytes for in vitro fertilization. In these cases, young females aged 3–5 weeks

(prepubertal females) are treated by injection of gonadotropin hormones and subsequently mated either to fertile or to vasectomized studs depending on the aim of the experiment. In practice, the females in question receive a first injection of 2.0–5.0 international units (IU) of the gonadotropin PMS (pregnant mare serum) in the afternoon of day 1, an injection that artificially induces a first estrus in the young females. 42–50 h later, they receive another injection of 2.0–5.0 IU of the gonadotropin HCG (human chorionic gonadotropin) that artificially induces ovulation.

Responses to gonadotropin injections vary from one strain to another, and, for this reason, the optimum doses and age of the mice to be injected must be determined, for each strain, by doing preliminary experiments[14] (Luo et al. 2011). Ovulation occurs approximately 12 h after the HCG injection, at which time the eggs (fertilized or not) can be collected by flushing the oviducts with a syringe filled with the culture medium. In the best experimental conditions the treated females can produce up to 30–40 embryos (hence the name superovulation), although the response to the hormonal treatment is highly variable between inbred strains (BALB/c is known to be a poor responder, while FVB is a high-responder). Female mice can also be superovulated after puberty, but in this case the production of embryos is much less efficient, presumably because the gonadotropin treatment interferes with the hormonal status of the treated female. If fertilized eggs are to be collected, it is important to mate no more than three or four hormonally treated females per male. The males should be older than 8 weeks and, ideally, proven breeders. Looking for vaginal plugs the following morning is then necessary to select the females that will be sacrificed to collect the embryos (at the desired stage). In order to be ready for the transfer of the manipulated embryos, it is essential to produce pseudopregnant females that will serve as recipients. This is typically achieved by mating outbred females (see Chap. 9) to vasectomized (sterile) males (created through a simple surgery). This mating is necessary for the uterine environment to become receptive, since only pseudo-pregnant females will allow the successful implantation and development of the fostered embryos. For more information and detailed protocols on these techniques, refer to the excellent manual by Nagy et al. (2003) and visit the webpage of the International Society for Transgenic Technologies (ISTT) at http://www.transtechsociety.org/.

2.2.3   Artificial Insemination

Several techniques for artificial insemination (AI) in the mouse have been described in the past (Wolfe 1967; Leckie et al. 1973). These techniques are simple and do not require sophisticated or expensive equipment. The sperm is taken from the vas deferens or the epididymis, mixed at room temperature in a

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[14] The response to gonadotropin injections may also vary from one batch of hormone to the next.

few milliliters of tissue culture medium, and injected directly into the uterus of the recipient female (at least 3×10^6 spermatozoa) using an insulin-type syringe, with a blunted needle, and a speculum to avoid harming the vaginal walls.[15] In this case, however, the vasectomized male must not be placed with the female before insemination, because the vaginal plug would interfere with the process. Capacitation of the spermatozoa does not seem to be a problem in this case.

Another technique has been reported where the sperm cells are injected directly into the upper uterine horns or the ampulla with a glass micropipette after laparotomy (uterine insemination) (De Repentigny and Kothary 1996). This second technique does not require such a high number of sperm cells, as compared to vaginal insemination.

Whatever the technique used, the yield in terms of embryo produced per inseminated female is quite low compared to other species. In spite of this low efficiency, artificial insemination has proven useful for obtaining hybrids between laboratory mice and mice of different species of the *Mus* genus (*Mus caroli* or *Mus cervicolor,* for example) because mice of some of these species do not copulate spontaneously with laboratory mice (West et al. 1977). Artificial insemination was also used for studying the possible mechanisms leading to segregation distortion in the progeny of males heterozygous for *t*-haplotypes[16] (Olds-Clarke 1989).

When given a choice, one must remember that F1 hybrids or outbred females have higher levels of fertility when used for AI. In addition, successful insemination can only occur when the inseminated female is in the late proestrus/early estrus stage.

AI will probably not be used very much in the future, because alternative techniques exist that are more reliable and have a much better yield.

2.2.4 In Vitro Fertilization in the Mouse

In vitro fertilization (IVF) is the most frequently used technology for assisted reproduction in humans. The technology was adapted to the mouse several years ago but this has not been easy to achieve and many critical steps had to be overcome (Whittingham 1968; Vergara et al. 1997). A major difficulty has been the development of suitable culture media allowing for a good rate of survival for the early mouse embryos. Another problem has been to optimize the timing of superovulation regimens for the different strains.

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[15] An ear speculum is an ideal tool. The extremity of a 20-ml glass pipette would also fit perfectly for this purpose.

[16] The *t*-haplotype is a small chromosomal region of chromosome 17 that is highly polymorphic among wild mice of the *Mus m. domesticus* species. Frequently, *t*-haplotypes of wild origin are not transmitted by heterozygous males in compliance with Mendel's laws (i.e., 50:50), but at a much higher frequency (95:5 or even 99:1).

Nowadays, protocols for IVF are available for most of the strains, even though some of them exhibit a higher rate of fertilization than others (Sztein et al. 2000; Nakagata et al. 2014).

The IVF technique generally consists of four steps: (i) young prepubertal females are injected with gonadotropins as described above; (ii) the morning following HCG injection (~8 h after), the oocytes are collected and gently washed; (iii) the oocytes are mixed for 4–6 h in vitro with either fresh or recently thawed frozen spermatozoa; and (iv) after inspection and selection, the fertilized eggs are transferred into a 0.5-day post-coitum (pc) pseudopregnant female. It is recommended to prepare the sperm sample one or two hours before mixing with the oocytes to allow capacitation to occur, although capacitation of mouse spermatozoa does not seem to be as crucial as it is in other mammalian species.

IVF is the technology of choice when it is desirable to rapidly expand a strain (for example, a transgenic line) from a few males that carry a desired or unique genotype, or for maintaining strains with poor breeding performance. IVF has the advantage that it can be performed using frozen or fresh sperm. The technique can also be used for the re-derivation of infected mouse colonies, and is frequently used for the transfer of genotypes of interest between laboratories.

2.2.5 Ovary Transplantation

When a mutant or transgenic female is potentially fertile (i.e., when it produces viable oocytes) but is unable to breed because of some kind of handicap, an ovarian transplantation is a good option. Some classic mutant mice such as *dystrophia muscularis* (*Lama2^{dy}*), *obese* (*Lep^{ob}*), and *dwarf* (*Pou1f1^{dw}*) were historically maintained by performing serial ovary transplantation. The technique consists of the surgical removal of the ovaries of the infertile donor female (even from very young females), and transfer into the ovarian bursa of an ovariectomized recipient female. Again, either freshly collected ovaries from the donor female or stored frozen/thawed ovaries can be used. Use of a recipient female with a different coat color from the donor is recommended to differentiate pups accidentally generated from residual ovary tissue (genotype of the recipient female).[17] Although the ovarian bursa is an immunologically privileged site, it is convenient to use recipient females that are histocompatible with the donor female. Alternatively, immunodeficient females (e.g., *nude* and SCID mutants) can be used as recipients.

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[17] It is for the rapid and safe identification of the origin of its progeny that mice of the strain 129/J segregate for the coat color alleles *Tyr^c* and *Tyr^{ch}*.

2.2.6 *Intra Cytoplasmic Sperm Injection*

Intra cytoplasmic sperm injection (ICSI, also known as *micro-insemination*) is another technology that is commonly used in humans to overcome persistent male infertility problems (for example, oligospermia, teratozoospermia, incapacity of the spermatozoa to pass through the zona pellucida, etc.). Here again, the technology has been adapted to the mouse with roughly the same basic protocol as in humans. In short, mature oocytes are held at the tip of a micropipette, by gentle suction, while a sperm head is injected deep into the cytoplasm of the oocyte by using a piezo-driven micromanipulator. This equipment and procedure allow for the safe injection of sperm heads by making only a very small hole in the zona pellucida that is promptly resealed once the needle is withdrawn (Ogura et al. 2003; Ogonuki et al. 2011). After the procedure, the oocyte is placed into an appropriate culture medium where its development is checked for a few hours.

ICSI has been adapted for use with immature (haploid) spermatogenic cells (round spermatids or elongated spermatids), and high rates of offspring development have been obtained (~30 % in some cases).

ICSI and ROSI (round spermatid injection) technologies have also demonstrated some practical advantages in the mouse. ICSI, for example, allowed for the recovery of normal pups from spermatozoa taken from the testes or epididymides of dead mice whose bodies had been stored at low temperature (between −20 °C and −80 °C) for a few years (Ogura et al. 2005; Ogonuki et al. 2006).

ROSI technology has also been cleverly used to reduce the time required for the development of fully congenic mouse strains by using the nucleus of round spermatids removed from young males (22–25 days of age) for the fertilization of superovulated oocytes flushed from 3-week-old females. With this technology, a backcross generation could be reduced to only 41–44 days, and a fully congenic strain (homozygous for 97.7 % of 176 tested markers) could be produced in 190 days (~6 months) (see Chap. 9) (Ogonuki et al. 2009).

2.2.7 *Cryopreservation of Mouse Embryos and Spermatozoa*

The mouse was the first mammal whose embryos were successfully frozen and stored at very low temperature. The methodology, which was published in 1972 (Whittingham et al. 1972; Wilmut 1972), required slow cooling (0.3–2 °C/min) and slow warming at some critical steps as well as the use of cryoprotectants to prevent ice crystals from damaging the cells of the embryo. In these initial experiments the cryoprotectants were either dimethyl sulfoxide (DMSO) or glycol. Since these pioneering experiments, the technique has been improved and nowadays mouse embryos are routinely stored at very low temperatures (in liquid nitrogen at –196 °C) for virtually unlimited periods and thawed when

requested with quite high rates of survival.[18] Embryo freezing and banking is achieved routinely in many laboratories, and is also available as a service from several commercial institutions. Short courses and demos with tutorials are available in several formats, for example as "webinars" or highly didactic movies, and are freely available through the internet.

Vitrification is another method of cryopreservation that has been developed more recently. With this method the embryos are osmotically dehydrated and then cooled by a rapid transfer into liquid nitrogen.

Cryopreservation of mouse spermatozoa has proved capricious for a long time and its rate of success is still relatively strain-dependent; for example, C57BL/6 sperm is difficult to freeze and the proportion of unviable sperm cells after thawing is quite high. However, the technology is rapidly improving and it is likely that most of the technical problems that still remain nowadays will be adequately solved in the near future (Sztein et al. 2000; Nishizono et al. 2004; Nakagata et al. 2014).

Freezing embryos and spermatozoa both represent a safe and (relatively) cheap way of exchanging mouse strains between different laboratories across the world. This practice has the advantage of reducing the risk of transmission of infectious diseases, a great concern for most veterinarians in charge of laboratory animal facilities.

Ovarian cryopreservation has been demonstrated to be another valid option for banking mouse genetic resources; in particular, it is the only technique that can be used to preserve oocytes from aged or problematic female breeders (Sztein et al. 2010).

Readers who are interested in the practice of cryopreservation technologies can refer to comprehensive reviews on the subject by highly experienced authors (Glenister and Rall 2000; Sztein et al. 2010; Nakagata 2011; Mochida et al. 2011). A didactic movie is also freely available on the internet: see reference list.

2.2.8 Twinning in the Mouse

The existence of the spontaneous occurrence of identical twins in the mouse is still debated. According to Grüneberg (1952), twinning occurs in the mouse as in many other mammalian species, but extremely infrequently; and twins may experience a disadvantage during their early embryonic life. Identical twins have been

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[18] Experiments performed at the Harwell (MRC) Research Centre have demonstrated that the damage caused by radiation (cosmic rays) to mouse embryos when stored at low temperatures for very long periods is practically negligible.

occasionally observed in utero at very low frequency, between embryonic days 8 and 10, but such embryos have not been recorded by embryonic day 16–17.[19, 20, 21]

McLaren and colleagues, looking for identity by DNA fingerprinting (using human minisatellite probes) in litters segregating for ten genetic loci, did not find any evidence of twinning in a population of 2,000 outbred mice. The authors concluded that twins are either extremely rare in the stock of mice they studied, or that they have such reduced viability that their chance of surviving to weaning is low (McLaren et al. 1995).

Spontaneous twinning is uncommon in the mouse; however, the experimental production of monozygotic twins by embryo splitting has been successfully achieved in several laboratories. Illmensee and colleagues demonstrated that in vitro splitting of mouse embryos at the 2-, 4- and 8-cell stage, followed by their transfer into empty zonae pellucidae, could be achieved with a relatively high rate of success. Embryonic development was monitored after in vitro culture for a few days and twin blastocysts from 2- and 4-cell splitting showed well-developed colonies with trophoblastic cells and clusters of inner cell mass (ICM) cells (Kaufman and O'Shea 1978; Illmensee et al. 2005).

2.2.9 Cloning Laboratory Mice

Cloning is an asexual method of reproduction that is commonly used in plants (e.g., cutting or striking) as well as in some insects: it offers the possibility of obtaining a potentially unlimited number of genetically identical individuals. In mammals, clones have also been produced experimentally by embryo splitting. More recently, cloning has been achieved by the experimental replacement of the nucleus of an unfertilized oocyte by the nucleus of a specific somatic cell from the same species, a process known as somatic cell nuclear transfer (SCNT). In most species, these experiments have been very difficult to perform, with low rates of success. Beyond these difficulties, many clones have developed severe pathologies that in many cases have undermined the interest of the enterprise. Cloning is no easy endeavor and many fundamental questions regarding possible modifications at the genome level during the early stages of development still remain to be understood.

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[19] It is not easy to observe twins by the mere examination of the implants in the mouse uterus, as placental fusion is frequent in this species.

[20] Discordances between the number of implants (dead or alive) and the number of corpora lutea does not support the idea that twinning commonly occurs in the mouse.

[21] Twinning (sometimes called "polyembryony") is the rule in nine-banded armadillos of the South American species *Dasypus novemcinctus*. In this species, the females regularly deliver progenies composed of four monozygotic twins. This regular production of genetically identical offspring makes the species a valuable model for multiple births.

Cloning the laboratory mouse has also been relatively difficult to achieve for technical reasons. Nonetheless, cloned mice were produced for the first time after the transplantation of nuclei taken from cells of the *cumulus oophorus*, hence the name of the first cloned female mouse: "*Cumulina*" (Wakayama et al. 1998).[22] Since then, mice have been cloned from a variety of different donor cells, including fibroblasts (tail skin), olfactory sensory neurons, ES cells, bone marrow cells, and liver cells. Recently, live mice have also been obtained after transplantation of the nucleus of peripheral blood leukocytes into enucleated oocytes from a drop of blood (Kamimura et al. 2013). Mice cloned from cumulus cell nuclei have even been themselves cloned in series for 25 generations, producing over 500 viable, fertile, and healthy clones derived from the original (single) donor. These experiments proved that serial recloning over multiple generations is possible in the mouse (Wakayama et al. 2013).

Compared to the situation in other species, in particular domestic species, the cloning of mice has relatively limited applications. This is because it is very easy in this species to obtain large populations of mice with exactly the same genotype. For example, mice of an inbred strain or born from a cross between two inbred strains are all genetically alike exactly as if they were cloned individuals (same genotypes). In these conditions, cloning mice may only help to enhance our understanding of the technical and biological factors that contribute to successful cloning in a species of economical interest. Experimenting with mice, biologists may be able to understand how the donor nucleus taken from a differentiated cell becomes reprogrammed by the oocyte cytoplasm to enable it to give rise to the different cell types. Similarly, the cloning of mice may help in the understanding of the reversibility of epigenetic changes occurring during tissue differentiation.

2.2.10 Mosaics and Chimeras

The terms *mosaic* and *chimera* are frequently incorrectly used in the scientific literature, even under the signature of professional geneticists. Mosaics are organisms composed of cells with a different genetic constitution, although deriving from one single conceptus (embryo). For example, an organism composed of cells with a different karyotype is a typical mosaic when this results from the loss or abnormal disjunction of a chromosome during the many mitoses that occur throughout embryonic development. An abnormal disjunction generates daughter cells with $2n - 1$ chromosomes and cells with $2n + 1$ chromosomes, and these cells are themselves mixed with normal $2n$ cells in variable proportions.[23] Such "chromosomal mosaics" are often viable, especially if the mosaicism concerns the

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[22] Cells of the *cumulus oophorus* are ovarian (but somatic) cells. They surround the oocyte and are shed with it upon ovulation.

[23] Cells with $2n + 1$ chromosomes (trisomic) are in general more viable than cells with $2n - 1$ chromosomes (monosomic).

X-chromosome or a minority of cells (see Chap. 3). 2n/3n and 2n/4n *mixoploid* mosaics have also been described in several mammalian species, including the mouse.

Mosaic organisms composed of normal cells and cells carrying a point mutation at a specific locus are probably very common (this point will be discussed in Chap. 7), but this mosaicism remains unnoticed if it has no deleterious consequences for the mutant cell. On the contrary, when spontaneous mutations accumulate in a cell (or group of cells) that provide the cell with the potential to divide indefinitely or to resist cell death, then these cells may become cancerous (malignant). In this sense, a mammalian organism affected by a cancer can be considered as a true mosaic since the malignant cells have indeed acquired a genetic constitution different from the non-malignant ones although they share the same origin.

Somatic (or mitotic) crossing-overs are yet another way of generating mosaic organisms, but only very few cases have been reported and documented in the mouse (Panthier et al. 1990). To conclude the definition of a mosaic, we note that, in general, mosaics are produced naturally, with no human intervention, while this is not the case with chimeras.

Chimeras (or chimaeras) are organisms that are composed of two (or more) different populations of genetically distinct cells (originated from different embryos), which generally result from human intervention. For example, an immunodeficient mouse that survives because it has received allogeneic bone marrow transplantation is a chimera, as is a mouse that results from the in vitro fusion of two or more morulae of different genetic origins (for example, from two different inbred strains). In this chapter, we will consider exclusively the case of chimeras resulting in a single complete mouse organism with pluri- or multiparental origin.

Mouse chimeras were created almost simultaneously in the early 1960s by Mintz, working at the Fox Chase Cancer Institute (Philadelphia, USA) and by Tarkowski, working at the University of Warsaw (Poland) (Tarkowski 1961, 1998; Mintz 1962). The first chimeric mice were produced by merging two independent morulae in vitro whose zona pellucida (oolemma) had been previously removed by a brief treatment with the enzyme pronase. These chimeras are referred to as *aggregation* or *allophenic* chimeras.[24] They developed in a chimeric animal of normal size, easily recognizable by a dappled coat color if the parental strains were appropriately selected (Mintz and Silvers 1967).

The aggregation technique developed by Mintz and Tarkowski was replaced in the early 1970s by a microsurgical technique that consisted of the injection of embryonic cells directly into the cavity of blastocysts (Gardner 1971).[25] This technique was later modified and improved in several ways (Brinster 1974; Mintz and Illmensee 1975; Papaioannou et al. 1975; Bradley et al. 1984; Stewart et al. 1994).

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[24] Sometimes called *tetraparental* chimeras.

[25] This cavity is often called a *blastocoel*.

Chimeric mice have been and still are important tools in biological research, as they allow us to answer questions related to cell lineage and cell potential with regard to tissue differentiation. By studying the muscles of chimeric mice constructed from two partner strains with different isocitrate dehydrogenase alleles ($Idh1^a$ and $Idh1^b$), it was demonstrated that the in vivo origin of the muscular syncytium is from myoblast fusion and not from repeated nuclear division in a non-dividing cell body (Mintz and Baker 1967).

Studying a series of hepatomas, which occurred in C3H/He $\times$ C57BL/6 chimeric mice, researchers found that most of these tumors were derived from cells of the hepatoma-susceptible C3H/He strain. However, they also observed that rare hepatomas were derived from cells of both genotypes, suggesting that some intercellular transmission of tumor information may have occurred or that the transformation might have occurred concurrently in two or more cells, indicating that hepatomas may therefore be genetically complex entities (Condamine et al. 1971).

Nowadays, chimeric embryos are produced routinely by injecting totipotent embryonic cells of different types (for example, embryonic cells from another embryo, embryonic stem (ES) cells that may or may not be genetically engineered, embryonic germ (EG) cells, etc.) into the blastocoel of recipient embryos. After this injection, the cells of the ICM of the recipient embryo merge with the transplanted cells and a chimeric embryo eventually develops to term. Today, the technique is mostly used for introducing a new genotype (that of the engineered ES cells) into the germ line of a chimera, allowing it to be ultimately materialized in a living mouse.

Another technique consists of using tetraploid embryos (which are artificially made by electrofusion of two 2-cell diploid embryos) as recipients for the engineered ES cells. It has been observed that, in this case, only the diploid cells (the ES cells) contribute to the formation of the neonates' body, while the cells derived from the tetraploid embryo will exclusively give rise to the trophectoderm and primitive endoderm. This technique is known as tetraploid complementation and, although not used extensively, it has been successfully used to create mice entirely derived from induced pluripotent stem cells (iPSCs) (Kang et al. 2009).

Another very clever technique resulting in 100 % germline transmission from competent injected ESCs has been developed. This technique consists of using a F1 host embryo (designated the "perfect host" or PH) which selectively ablates its own germ cells via tissue-specific induction of diphtheria toxin. This approach allows competent microinjected ES cells to fully dominate the germline, eliminating competition for this critical niche in the developing and adult animal (Taft et al. 2013).

Although chimeras can be either male or female, in experience the majority is male because most of the ES cell lines are XY. Having male chimeras is actually good because they generally have good germline transmission (Nagy et al. 2003). Tetraparental chimeras can breed if the two embryos at the origin of the chimera are both of the same sex. If this is not the case, for example if one set of cells is genetically female and the other genetically male, intersexuality (and sterility) often results. Even when the two embryos that participate in the formation of the chimera are of the same sex, the fertility sometimes depends on which

cell line gave rise to the ovaries or testes. For this reason, the association of a tetraploid (4n) partner with a diploid (2n) one, as explained above, is particularly advantageous.

The production of allophenic chimeras has been used in various contexts to answer biological questions that would not have been easily answered otherwise. For example, chimeras have been produced to transmit lethal genes in the mouse and to demonstrate allelism of two X-linked male-lethal genes, *jp* and *msd* (Eicher and Hoppe 1973). In another example, viable aggregation chimeras have been made by merging normal embryos with embryo homozygous for the recessive lethal mutation Hairy ears (*Eh*-Chr 15), which indicated that the mutation in question was not cell-lethal (we now know that it is a large deletion) (Guénet and Babinet 1978). Finally, especially noteworthy is the production, by Kobayashi et al. (2010), of the first viable rat–mouse chimeras. In this report, the authors also demonstrated that rat iPS cells could rescue organ deficiency in mice, opening new frontiers for tissue engineering.

2.3 Basic Notions of Genetics

2.3.1 Genes and Alleles

In his famous note reporting the results of his *Experiments on Plant Hybridization* (1866), Mendel alluded to "*factors*" or "*units of inheritance*" that are transmitted from one generation to the next and determine the phenotypic characteristics of plants. Using such words, it is clear that Mendel was referring to the concept of genes, but he did not coin any specific word to define these "*units of inheritance*". Several years later, in 1889, de Vries published a book entitled *Intracellular Pangenesis* in which he led an interesting discussion concerning the "*units*" or "*particle bearers of hereditary characters*", and he recommended that the word pangens be used to specify these particles (de Vries 1910). Finally, it was the Danish biologist Johannsen who proposed, in 1909, that the (Danish) word "*gen*" be used to describe the units of heredity. The same Johannsen also introduced the terms *phenotype* and *genotype,* and almost at the same time Bateson proposed the term *genetics* to describe the science dealing with *gens* (*genes*).

Shortly after the confirmation that DNA was the molecular basis for inheritance (seminal work published by Avery, McCarty, and MacLeod in 1944), the definition of the gene was translated into molecular terms and became "a segment of DNA of variable size encoding an enzyme". This was in compliance with the famous "one-gene-one-enzyme" theory formulated by Beadle and Tatum.[26] This definition was reconsidered when it was recognized that some proteins are not enzymes. The

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[26] G.W. Beadle and E.L. Tatum were awarded the Nobel Prize in Physiology or Medicine in 1958 for their discovery of the "*role of genes in regulating biochemical events within cells*".

motto was then changed to "one-gene-one-polypeptide" and the definition of the gene was modified accordingly.

In 2002, once the sequencing of the mouse nuclear DNA was completed, followed by the extensive analysis of the transcriptome[27] and the confirmation that a great number of genes were not translated into polypeptides, the definition of the gene changed again. Nowadays a gene corresponds to a segment of DNA that is transcribed into RNA. Some of these RNA molecules, the messenger RNAs (mRNAs), are translated into polypeptides while many others are not translated but have nevertheless important functions as RNAs (see Chap. 5).

Recently, information collected from the systematic analysis of a single transcriptome revealed that mammalian DNA is pervasively transcribed from both strands, and that the proportion of DNA transcribed into RNAs is much greater than expected. The same analysis also revealed that mammalian genes are not always clearly individualized in the DNA strands; on the contrary, their limits are often difficult to define, with some small genes being nested into larger ones, inserted for example in the introns. Thus, it is seems clear that the concept of the gene will have to be reconsidered and its definition reformulated in the near future. For the time being, we will stay with the idea that a gene is a functional unit materialized in a short segment of DNA, which is transcribed into RNA, and whose inheritance can be followed experimentally generation after generation.

For decades, the *genome* was known as the collection of genes of a given species. The word was coined at the beginning of the twentieth century, and at that time it was used to exclusively define the collection of genes. Nowadays, the concept includes both the genes (i.e., the coding sequences) and the sequences of DNA that are intermingled with the genes and are themselves heterogeneous. Thus, when they refer to the mouse genome sequence, geneticists in fact refer to the sequence of the whole nuclear DNA.

The number of genes in the mouse genome is expected to be in the range of 25,000–30,000 but, for reasons that will be discussed further, this assessment is not accurate and will probably never be. For some genes, the number of copies in the genome varies across the different strains, or even individuals, and many among these genes are non-functional (see Chap. 5 regarding CNVs and pseudogenes). It is also known that some genes are present in some strains (or species) and absent in others. All these variations, of course, hamper the accurate evaluation of the number of genes.

In addition to these inter-strain quantitative variations in the number of genes, we know that several different RNAs (coding and non-coding) can be transcribed from the same gene by the mechanism of alternative splicing (detailed in Chap. 5), and this tremendously increases the number and diversity of the molecules potentially encoded in the genome. Obviously, it is the inventory and classification of all these transcripts that would be important to make, rather than an accurate

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[27] The transcriptome corresponds to the full set of RNA molecules that are transcribed from the genome. This point will be extensively discussed in Chap. 5.

assessment of the number of genes. This goal is certainly in the minds of many geneticists, but it is a serious challenge and is difficult to achieve.

Whatever the actual number of genes in the mouse genome, once a gene is biologically defined either in terms of function or structure, it can be precisely localized on a specific chromosome of the species using a variety of techniques. The position of such a gene defines its *locus* (plural *loci*, the Latin word for "place") and we will extensively discuss the strategies used for the localization of the genes in Chap. 4.

Many genes exist in several versions (variants) called *alleles*. The word "allele" is an abbreviation of the ancient word *allelomorph*, which was used in the past to describe the different forms of a gene, detected as different phenotypes. Formerly, the concept of alleles was tightly associated with the concept of mutation producing a phenotypic variant different from the wild type (i.e., the version most commonly found in wild animals). In this case, the new version of the gene was defined as a *mutant allele* and was identified in mice, for example, by a different coat color, a heritable skeletal defect, or a debilitating neurological disease.

The concept of the allele has also progressively changed and nowadays one can say that any change at the DNA level that translates into a phenotype different from the previously known phenotypes defines a new allele, regardless of whether the phenotype associated with the new allele is deleterious. The substitution of a nucleotide in a coding sequence that leads to a change in the global electrical charge of a protein characterizes a new allele because, even if the function of the protein is not affected, one can distinguish by electrophoresis the new protein from the other proteins encoded by the same gene: it is a different phenotype.[28] If the nucleotide substitution modifies the activity of the protein, with deleterious consequences, in this case the new allele is either a *hypomorphic* or *null allele* (see Chap. 7).

Other types of structural variations at the DNA level (for example, the so-called single nucleotide polymorphisms or SNPs) can also be used to distinguish allelic variants (DNA variants in this case), even if these allelic variants do not confer any phenotypic change on the animal. In these conditions the reader may appreciate how the definition of the word allele has evolved with time. In the past, the function of the protein, assessed by its effect on the phenotype of the animal, was crucial to define a new allele. Nowadays, any structural change that can differentiate a gene from another at the same locus defines an allele, regardless of the phenotypic consequences. We will come back to this point when discussing the genetic markers used for gene mapping (Chap. 4).

According to the Mouse Genome Database (as of November 2014), 10,425 genes of the mouse have at least a mutant allele and the mouse genome comprises 40,713 alleles altogether. The whole collection of alleles that are segregating in a given population represent what geneticists call the *genetic polymorphism*. This

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[28] The word *electromorph* has been coined to define the alleles characterized by a different global electrical charge.

notion of polymorphism applies to the series of alleles at a specific locus or to all loci of a strain or species.

In the mouse, the gene encoding tyrosinase (*Tyr*), an enzyme that is instrumental in the synthesis of the pigment melanin, was one of the first (if not the first) to be identified based on a variation in coat color. At the *Tyr* locus, one allele encodes a normal, functional tyrosinase, and the other encodes a non-functional enzyme resulting in albinism. Nowadays, over 120 different mutations have been collected at the *Tyr* locus, some of them having a phenotype affecting coat color (for example, chinchilla-*Tyr^{c-ch}*, extreme-dilution-*Tyr^{c-e}*, hymalaya-*Tyr^{c-h}*, to cite a few). However, it is likely that sequencing will identify many others that are not yet detected because they have no obvious phenotype. Such a collection of a series of alleles always represents an interesting resource for geneticists.

The Mouse Genome Database specifies rules and guidelines for mouse and rat gene nomenclature (http://www.informatics.jax.org/mgihome/nomen/gene.shtml), with which it is extremely important to comply because genetic nomenclature is a universal language. We recommend that readers frequently refer to these guidelines, which are presented in a very didactic form with many different examples. In case of doubt, information may also be requested directly from the staff of curators, as explained on the website.

2.3.2 Allelic Interactions

When the alleles at a given locus are the same on both chromosomes, the mouse is *homozygous* and the phenotype that characterizes the allele in question is fully expressed: the situation is simple. When the two alleles are different, the mouse is *heterozygous* and the phenotype depends upon the interactions between the two alleles. To illustrate the situation, we will again consider a gene we are already familiar with: the gene encoding tyrosinase (*Tyr*-Chr 7). As we already mentioned, this gene has several alleles, among which some are non-functional; this is the case with *Tyr^c*. When a mouse has the *Tyr^c* allele on both chromosomes 7 (homozygous), it is albino. In contrast, when the mouse has a non-functional allele on one chromosome 7 and a functional allele on the other chromosome, it is heterozygous and is pigmented just like a wild mouse. The *Tyr^c* allele is said to be *recessive* and the normal allele, or wild-type allele (*Tyr^+* or sometimes only +), is *dominant*. In this case, the lack of functional tyrosinase is completely compensated for at the cellular (melanocyte) level by the presence of a single copy of the normal (wild-type) allele.[29]

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[29] When an allele is fully dominant, geneticists often write the genotype *Mut/–*, indicating that the allele in question completely determines the phenotype.

Some other alleles at the *Tyr* locus have less dramatic effects than Tyr^c on the synthesis of the pigment melanin and in many cases the mice are pigmented, although always less than or differently from the wild type. For example, mice homozygous for the extreme dilution $Tyr^{c\text{-}e}$ allele appear "*slightly stained or dirty black-eyed white*" (Detlefsen 1921). They have a light grey coat color, almost white, but their eyes are solid black, unlike albino mice. Mice homozygous for the chinchilla allele $Tyr^{c\text{-}ch}$ have a diluted coat color (they really look like chinchillas—hence the name of the mutant allele) but their coat color is much darker than mice homozygous for $Tyr^{c\text{-}e}$. Finally, mice homozygous for the Himalayan allele $Tyr^{c\text{-}h}/Tyr^{c\text{-}h}$ have a remarkable pattern of pigmentation with a mainly white body and light-ruby eyes and only the tip of the nose, tip of the ears, and the tail are normally pigmented (black). This is because the tyrosinase encoded by the $Tyr^{c\text{-}h}$ allele is active only in the colder parts of the body, where the temperature is below 35 °C (the enzyme is heat-labile or thermo-labile). This is the same phenotype observed in Siamese cats.

With so many alleles at our disposition, we could breed a wide variety of mice heterozygous or homozygous for different alleles and we would then discover that the normal allele (Tyr^+) is dominant over all other alleles. However, if we grade the phenotypes of the mice based on the decreasing intensity of the coat color for all the possible combinations of the four alleles at the *Tyr* locus- Tyr^+; $Tyr^{c\text{-}ch}$; $Tyr^{c\text{-}e}$ and Tyr^c we observe that they display an almost continuous gradient of pigmentation from type to albino (i.e. $Tyr^+/-$ > $Tyr^{c\text{-}ch}/Tyr^{c\text{-}ch}$ > $Tyr^{c\text{-}ch}/Tyr^{c\text{-}e}$ > $Tyr^{c\text{-}ch}/Tyr^c$ > $Tyr^{c\text{-}e}/Tyr^{c\text{-}e}$ > $Tyr^{c\text{-}e}/Tyr^c$ > Tyr^c/Tyr^c) (from Silvers 1979). The observation of intermediate phenotypes such as $Tyr^{c\text{-}ch}/Tyr^{c\text{-}e}$ or $Tyr^{c\text{-}e}/Tyr^c$ allows for the definition of another kind of allelic interaction that is called *incomplete dominance* or *intermediate dominance*, or sometimes *partial dominance*. In these cases one allele is not completely dominant over another, and the expressed physical trait is in between the dominant and recessive phenotypes. In this context, the phenotype of mice homozygous for the Himalayan allele $Tyr^{c\text{-}h}$ cannot be considered as "intermediate"; they are simply different and unique.

The series of alleles that we described at the *Tyr* locus is common in plants and vertebrate species, and many other examples are available in the mouse. As we already said, in most cases the wild-type allele, the one that is most frequently found in wild mice, is often dominant over all other alleles at the same locus; but this is far from being a rule. At the Agouti locus (*A*-Chr 2), where there is another long series of alleles (over 400) affecting coat color, the wild-type allele agouti (*A*) has an intermediate position: it is dominant over some alleles like black-and-tan (a^t), non-agouti (*a*), or extreme non-agouti (a^e), but it is recessive to yellow (A^y), viable yellow (A^{vy}) and a few other *A* alleles. By the way, it is interesting to note that the yellow allele (A^y) in question, although dominant over *A* if we consider the coat color, is nevertheless a recessive lethal when homozygous (see Fig. 1.1). A^y/A mice have a beautiful yellow coat color but A^y/A^y embryos display characteristic

abnormalities at the blastocyst stage and die on the sixth day of gestation.[30] This observation means that the notion of dominance and recessivity must be considered only in the context of a specific phenotype.

True dominant mutations, i.e., mutations for which the phenotype of the heterozygote ($Mut/+$) is indistinguishable from the phenotype of the homozygous mutant (Mut/Mut), are rare in the mouse and in mammals in general. In most instances, the dominant alleles behave just like the yellow (A^y) allele and are lethal when homozygous. Among the few exceptions are some keratin mutant alleles such as Rex ($Krt25^{Re}$), Caracul ($Krt71^{Ca}$), and possibly a few others such as the coat color mutation Sombre ($Mc1r^{E\text{-}so}$) and the neurological mutation Trembler ($Pmp22^{Tr}$).

Another type of allelic interaction that is extremely common in mammals is *co-dominance*. Co-dominance is when the two alleles at a given locus are both expressed in the phenotype of the heterozygote, which has a phenotype of its own. In most genetics textbooks the concept of co-dominance is exemplified by the AB blood groups in humans, where the AB heterozygotes have a phenotype in which both the A and B antigens are expressed on the red blood cells. Blood groups homologous to the human AB system do not exist in the mouse, but practically all the genes expressed in the form of proteins with different electric charges are co-dominantly expressed. Glucose-6-phosphate isomerase (symbol *Gpi1*-Chr 7) is an enzyme that is expressed in most cells; it catalyzes the conversion of glucose-6-phosphate into fructose-6-phosphate. Several alleles at the *Gpi1* locus have been characterized, of which four are common, viable and functional: $Gpi1^a$ and $Gpi1^b$ are found in laboratory inbred strains, $Gpi1^c$ is a spontaneous mutation of recent occurrence in the BALB/c inbred strain, and $Gpi1^d$ was discovered in wild mice. It is likely that many more alleles (electrophoretic variants) exist in wild mice and have not (yet) been identified. All these alleles are co-expressed in mice heterozygous at the *Gpi1* locus.

When the phenotypes of the different alleles at a given locus are carefully analyzed, interesting observations can be made concerning the allelic interactions. A good example is the case of the locus encoding the enzyme argininosuccinate synthetase (ASS). At this locus, several mutant alleles have been identified in the mouse that are potentially interesting models for the human disease citrullinemia type I (CTLN1, OMIM# 215700). Among all the hypomorphic alleles, two are more interesting than others: A*ss1*^{bar} and A*ss1*^{fold}, because they faithfully replicate the pathology observed in human patients suffering from CTLN1, with variations in terms of survival rate, developmental delay, and neurological phenotype. Homozygous and compound heterozygous combinations of the two alleles create

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[30] These yellow mice posed a problem to Cuénot while he was trying to demonstrate that Mendel's laws also apply to mammals. When intercrossing A^y/A mice, he did not find the expected 1:2:1 proportions of phenotypes for a single gene with two alleles, but instead found a 1:2:0 ratio. However, Cuénot provided the correct explanation for these "unusual" proportions.

a spectrum of severe ($Ass1^{bar}$/$Ass1^{bar}$), intermediate ($Ass1^{fold}$/$Ass1^{fold}$), and mild ($Ass1^{bar}$/$Ass1^{fold}$) phenotypes. However, the observation that the compound heterozygotes, carrying one severe allele ($Ass1^{bar}$) and one mild allele ($Ass1^{fold}$), exhibited a milder phenotype (including residual activity of liver ASS and less pronounced plasma ammonia levels) than mice carrying two copies of the mild allele ($Ass1^{fold}$/$Ass1^{fold}$) was quite unexpected. Obviously, this warrants further investigation concerning the molecular interactions between the different ASS1 mutant proteins (Perez et al. 2010).

Dominance, recessivity, and co-dominance are the most common forms of allelic interactions but there are also a few others that deserve, at least, a short comment. *Semi-dominance* has been used to characterize mutant alleles where the phenotype of heterozygotes is different (and often intermediate) from both kinds of homozygotes. A typical example is the Kit^{W-f} allele at the *Kit* locus (Chr 5), where Kit^{W-f}/+ heterozygous mice have a light grey coat with a spot on the belly and a small blaze on the forehead, while heterozygous Kit^{W-f}/Kit^{W-f} mice are extensively spotted (Guénet et al. 1979). Amazingly, the tails of these mice perfectly characterize the situation: the tail is normally (i.e., completely) pigmented from the base to the tip in wild-type mice; it is half pigmented in heterozygotes and it is completely unpigmented (white) in homozygotes. Another example is the semi-dominant spontaneous mutation called *Naked* (*N*) on distal chromosome 15 (Hogan et al. 1995). The semi-dominant allelic expression is common in the mouse but it is sometimes used for alleles that would be best characterized as incompletely dominant.

Overdominance is a rather rare condition in which the heterozygotes (*M*/*m*) have a phenotype that is more pronounced than that of either homozygote (*M*/*M* and *m*/*m*). We report such a case of overdominance in Chap. 6 with the *Callypyge* mutation in sheep. No similar mutation has ever been reported in the mouse, but some may exist. Overdominance is sometimes used as an alternative word for *superdominance*. Superdominance characterizes a situation where the heterozygotes have a selective advantage over homozygotes. This is the case, for example, with the human allele that encodes sickle-cell anemia (HBB[s]) or drepanocytosis. In the countries where malaria is endemic the gene encoding the defective hemoglobin, although lethal when homozygous, is present in over 40 % of the population, while we would expect it to be strongly counter-selected. This is because the HBB[s] allele confers resistance to malaria in the heterozygotes. Homozygotes with the normal allele get sick and sometimes die when infected by *Plasmodium*; homozygotes for the mutant allele also get sick from drepanocytosis but the heterozygotes survive *Plasmodium* infection and do not develop severe anemia. This selective advantage of a specific combination of alleles is probably also found in wild mouse populations but, to date, it has never been described in any laboratory mouse or rat population.

In this review of the different forms of allelic interactions, one must not forget the case of genes that are X-linked. In mammalian species, the males have only one X-chromosome and, in these conditions, the individuals are *hemizygous* for all the genes carried by this chromosome and all are fully expressed. In females, the situation is more complex and the situation will be discussed in full detail in

Chap. 6. Without going into detail, one can say that due to the phenomenon of X-inactivation, which is a mechanism of dosage compensation operating in female mammals, most X-linked genes are functionally haploid and only one copy of every gene is transcribed, while the other copy is switched off. The inactivation of one allele over the other is, in most cases, a random process. In the mouse, a few genes are in the so-called pseudo-autosomal region of the X-chromosome and behave as autosomal genes. The gene encoding steroid sulfatase (*Sts*) is one example.

When a mutation occurs in a mouse population, the allelic interactions exhibited by the novel allele is important information to take into account in the process of genome annotation. If the novel allele is dominant or semi-dominant, it makes sense to guess that the observed phenotype is the consequence of a structural defect of the protein encoded by the mutant allele. On the contrary, when the novel allele is fully recessive, this would indicate a loss-of-function (or hypomorphic) mutation for the protein encoded by the mutant allele. For example, mutations in the genes encoding collagens or fibrillins, which generate a structural defect in the proteins in question, are almost always dominant or semi-dominant.[31] On the contrary, mutations that cause an "*inborn error of metabolism*", as Garrod used to designate some metabolic diseases, are usually recessive. In fact, there is some logic in these observations: the genes encoding metabolic enzymes are in general *haplo-sufficient* (50 % of normal levels are sufficient to complete the metabolic function), while the situation is radically different if the encoded polypeptide is involved in the differentiation of a specific tissue.

2.3.3 Epistasis and Pleiotropy

Many phenotypic traits are controlled by more than one gene, and, conversely, it is relatively common to observe that a given gene contributes to the phenotypic expression of one or several other genes. In the forthcoming chapters (in particular in Chap. 10, which is devoted to quantitative genetics) this point will be considered in detail. For the time being, we will just discuss a few examples that will help introducing two fundamental notions in genetics: *epistasis* and *pleiotropy*.

2.3.3.1 Epistasis

Epistasis characterizes a situation where the phenotypic expression of a gene (or allele) *A* depends on the presence, at some other loci in the genetic background (*B*, *C*, *D*), of one or several specific alleles that modify or suppress the classical

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[31] A mutation that leads to the synthesis of a mutant protein that interferes or disrupts the activity of the wild-type protein in the multimer is called a *dominant-negative mutation*. A typical example is found in the syndrome of osteogenesis imperfecta (O.I. Type III) in which structurally defective type I collagen is formed.

phenotype of gene *A*. To put it in other words: epistasis is an interaction between non-allelic genes in which one gene suppresses or enhances the expression of another. The gene that is expressed is *epistatic* over the others genes, which are themselves *hypostatic*. Once more, the genes that are involved in the development of mouse coat color offer simple and didactic examples.

Exploiting the variety of alleles at the five major loci governing the mouse coat color (Agouti-*A*; Tyrosinase-*Tyr*; Brown-*Tyrp1*; Dilute-*Myo5a*; and Pink-eyed dilution-*Oca2*) one can generate a large collection of mice with a wide array of coat colors. However, sometimes it happens that the effects of a given mutant allele cannot be observed if another allele is present in the genome of the same animal. A mouse with a non-agouti, brown coat color (genotype *a/a*; *Tryp1^b/Tyrp1^b*) would appear "chocolate", unless the *Tyr^c* allele (which is at the *Tyr* locus on a different chromosome) is homozygous. In this latter case, the mouse would appear albino—i.e., completely white, and this is because the *Tyr^c* allele exhibits epistatic interaction with all other coat color genes. We know the reason: it is because the *Tyr^c* allele is non-functional. Thus, there is no tyrosinase synthesis, no melanin, and no pigment, be it black or yellow.

Another example of epistatic interaction in the mouse is between the *Mc1r^e* allele (recessive yellow-Chr 8) at the locus encoding the melanocortin 1 receptor and the *Mlph^{ln}* allele (leaden-Chr 1): mice with a *Mc1r^e/Mc1r^e*; *Mlph$^+$/−* genotype have a deep yellow coat color, and mice with a *Mc1r$^+$/−*; *Mlph^{ln}/Mlph^{ln}* genotype have a light gray coat color, like diluted, but these mice are indistinguishable from the *Mc1r^e/Mc1r^e*; *Mlph^{ln}/Mlph^{ln}* mice. In short: *Mlph^l* is epistatic to *Mc1r^e* and the phenotypic effects of the recessive yellow allele are entirely suppressed by the phenotypic effects of leaden (Hauschka et al. 1968). Many mutations affecting enzymes of cellular metabolism exhibit epistatic effects, especially when they are in the same metabolic pathway.

Epistatic interactions are common with traits governing quantitative inheritance: the quantitative trait loci (QTLs). A heritable quantitative trait can be under the control of several genes with additive effects, the genes in question having different strengths in the determinism of the phenotype. When two alleles at different loci have a stronger effect on the phenotype than each allele individually, this is referred to as *synergistic epistasis*. The opposite situation also exists and is called *negative* or *antagonistic epistasis*.

When we described the epistatic effects of the *Tyr^c* (albino) allele on the expression of all other genes involved in coat color determinism, we assumed that this effect was the direct consequence of the expression (or non-expression, in this case) of the protein encoded by the gene (tyrosinase). The situation is sometimes much more subtle. For example, the mutant allele *Apc^{Min}* (at the adenomatous polyposis coli gene-Chr 18) is a dominant allele (although recessive lethal) that predisposes mice to the development of multiple intestinal neoplasia (Moser et al. 1990). However, some mouse strains are much more susceptible to this syndrome than others. Mice of the strain C57BL/6, for example, are severely affected and develop many intestinal tumors, while mice of the AKR strain, with the same

Apc^{Min} allele (congenic mice), develop only a few tumors. This dramatic phenotypic difference between the two inbred strains has been found to be the consequence of an epistatic interaction between the *Apc^{Min}* allele and another gene called *Modifier of Min* encoding a phospholipase A2 (*Pla2g2a*-Chr 4), itself with two alleles: *Pla2g2a^{Mom1-r}* and *Pla2g2a^{Mom1-s}*. However, the *Pla2g2a* alleles have a phenotypic effect only when the *Apc^{Min}* allele is in the same genome. In other words, *Pla2g2a* is a modifier gene whose phenotypic expression is conditional to the presence of the *Apc^{Min}* allele. Such situations are very common in the mouse, and the *Apc^{Min}* allele has several other independent modifiers (Dietrich et al. 1993). The identification and study of modifier genes opens interesting avenues for unraveling the networks that determine robustness and resistance to certain diseases. Hence, we emphasize the importance of the use of pure inbred backgrounds in mouse models (see Chap. 9).

2.3.3.2 Pleiotropy

Pleiotropy describes a situation that is in fact extremely common in genetics: it simply means that a mutant allele has an effect on different phenotypic traits. In fact, if we carefully analyze most of the mutants with a deleterious phenotype, we would then discover that almost all of them in fact exhibit a panel of different phenotypes. The yellow allele (A^y) was identified because of its eye-catching phenotype, with a beautiful yellow coat color, but the mutant mouse exhibits many other phenotypes. For example, the mice are slightly diabetic, exhibit liver hypertrophy, and many become obese and sterile after the first few months of life. They are also more susceptible to several kinds of tumors than normal mice and are more aggressive.

If we consider that the products of most genes are involved in several cellular functions, pleiotropy seems to be more the rule than the exception. It simply means that the gene in question codes for a product that is used by various cells, or has a signaling function on various targets, or that the protein is an enzyme or a transcription factor that is involved in several pathways.

2.3.4 Penetrance and Expressivity

2.3.4.1 Penetrance

Penetrance is a term used to express the fraction of individuals of a given genotype that effectively exhibit the expected phenotype. Penetrance is usually expressed as a percentage. For example, if a particular dominant mutation has 80 % penetrance, then 80 % of the mice carrying the mutant allele will develop the phenotype and 20 % will look normal (Fig. 2.1).
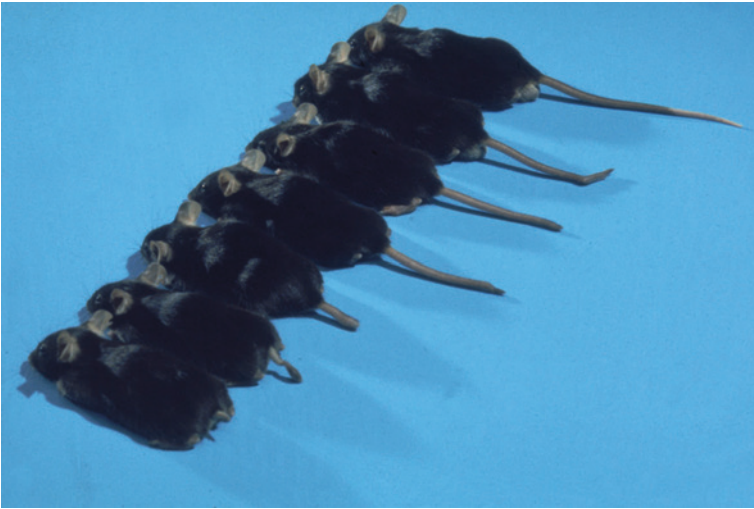
Fig. 2.1 *Penetrance and expressivity*. The figure illustrates the concepts of penetrance and expressivity. In this example, the mutation brachyury (*T*-Chr 17), affecting six out of the seven mice, exhibits great variations in expressivity; some mice have a tail longer than others, even if they all are clearly short-tailed. When a mouse with a short tail (genotype certainly *T/+*) is crossed with a normal mouse (+/+), the proportion of affected offspring is often lower than 50 %. Some mice have an extremely severe reduction of the tail, exhibit a *spina bifida*, and die at birth while others have an almost normal tail (normal overlaps). The penetrance characterizes the fraction of individuals of a given genotype that actually exhibit the phenotype typical of the mutant allele, irrespective of the degree of its expression. The expressivity characterizes the phenotypic variation among individuals having the same genotype. It is now well established that modifier genes influence the phenotypic expression of many mutant alleles. However, the action of these modifiers cannot explain all types of variations since phenotypic variations are also observed in inbred strains—as in the case illustrated here, where all the mice are from the same inbred strain. Variations in penetrance and expressivity are common for skeletal and eye mutations in all species

2.3.4.2 Expressivity

A genotype exhibits variable *expressivity* when individuals with that genotype differ in the extent to which they express the phenotype normally associated with that genotype. The best example illustrating the concept of expressivity and differentiate it from the concept of penetrance (which is not always easy) was provided by Danforth regarding a population of cats in Key West Island (a population also known as "Hemingway's cats"), in which a dominant mutation resulting in polydactyly is highly prevalent. Observing the cats in question, Danforth wrote, "*the polydactyly phenotype shows good penetrance, but variable expression*". This simply meant that a high percentage of cats indeed had extra toes, but the number and size of the extra toes varied from one animal to the next (Danforth 1947). Another example is the case of spotting in cattle. Observing a herd of cows of the Frisonne breed one may notice that, although all the cows are spotted (penetrance

is 100 %), the ratio black/white is highly variable from one cow to the next. The spotting is highly variable in shape (no surprise) but also in extension (which is more surprising). These are variations in expressivity of the spotting allele.

Although the examples we selected were from cats or cows, similar situations can be easily found in mice where mutations yielding extra digits and white spotting are common. In short, variable expressivity means that there is a large amount of phenotypic variation among individuals with the same genotype (Miko 2008).

The causes of penetrance and expressivity are not well understood. In the mouse, as well as in the rat, one can study the phenotypic expression of the same mutation in different genetic backgrounds and note more or less consistent differences, indicating the existence of a genetic component (modifier genes). However, in the same species, one can also observe phenotypic variations in animals having exactly the same mutation in exactly the same genetic background—meaning that genetic factors are not the only factors involved in the variation of penetrance or expressivity. In these conditions, it makes sense to consider that epigenetic factors or stochastic events are probably also at work. In Chap. 6, dealing with the epigenetic control of genome expression, we will discuss a situation where the coat color of mutant mice is strongly influenced by environmental factors.

Having control of the factors that determine expressivity is of major importance in human medicine, because many diseases with a genetic determinism (for example, cancers, neurological diseases, and skeletal abnormalities) often exhibit great variations in expressivity (Nadeau 2003).

2.4 Phenotyping Laboratory Mice: The Mouse Clinics

As we will explain in the chapters to come, researchers now have all the means and tools to create a great variety of alterations in the mouse genome; for example, they can switch off any gene they wish, and at any time. They can interfere (transitorily or not) with gene regulation, they can make all sorts of genetically engineered mice with cloned DNAs of their choice, etc. Of course, all of these alterations induced at the genome level are expected to result in changes at the phenotypic level in genetically modified animals, and the careful analysis of these phenotypic changes is obviously fundamental for the process of *genome annotation.*[32] However, the problem is that, though it is relatively easy to localize and precisely characterize a DNA sequence, especially nowadays, it is much more difficult to unambiguously establish the link between a DNA alteration and an abnormal phenotype. Examples are numerous where the knockout allele of a theoretically important gene was initially reported as having "no detectable phenotype," and this was to the great surprise (and sometimes to the disappointment) of its creator (Colucci-Guyon et al. 1994).

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[32] Genome annotation consists of attaching biological information to a particular DNA sequence, or of establishing a link between a gene (or a small chromosomal region) and a given phenotype by any possible means.

Phenotyping has become one of the main concerns of mouse geneticists over the last 10 years and, mainly for this reason, many laboratories and institutions have developed what is now called a "*Mouse Clinic*". In these clinics, mouse mutants or strains are thoroughly analyzed for the greatest possible number of parameters using a panel of highly standardized phenotyping protocols. In most cases the basic protocols are focused on behavior, bone and cartilage development, neurology, clinical chemistry, eye development, immunology, allergy, steroid metabolism, energy metabolism, lung function, vision and hearing, pain perception, molecular phenotyping, cardiovascular analyses, and gross pathology. For example, the International Mouse Phenotyping Resource of Standardised Screens (IMPReSS) contains standardized phenotyping protocols, which are essential for the characterization of mouse phenotypes (see https://www.mousephenotype.org/impress). The use of standard procedures and defined protocols allows data to be comparable and shareable, and even allows interspecies comparisons to be performed, which may help in the identification of mouse models of human diseases.

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