A method for gender determination in newborn dark pigmented mice

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In many studies using mice, investigators must determine pups' gender at a very early postnatal stage. The gender of mouse pups is typically assessed by measuring the anogenital distance, which is greater in males than in females. This method, however, has proven to be difficult and not completely reliable. The authors describe a quick, easy and reliable method to establish the gender of pigmented mice. In male mice, a pigment spot on the scrotum is visible to the naked eye from the first day of life onwards, whereas female pups lack visible pigmentation in the anogenital region. In lightly pigmented or albino mice, the pigmentation is not obvious or not at all visible. The authors show that identifying this pigment spot is a more accurate and efficient method of determining pup gender compared with measurement of the anogenital distance. This 'spot on' method would therefore be a useful adjunct to conventional methods for determining the gender of pigmented neonatal mice.

The mouse is a convenient animal model system that is used for a diverse range of scientific experiments. Genes and environments can be manipulated or controlled in mice, laboratory strains are easy to handle and maintain, and the relatively high fecundity of mice facilitates efficient breeding programs[1]. Pups can be generated rapidly, as the duration of the gestation period in mice is approximately 19–21 d, and the average number of pups per litter ranges between 5 and 10.

For most research using mice, it is essential to establish the gender of the experimental subject. In older mouse pups, the gender can be identified according to several characteristics. In female mice, for example, the nipples are clearly visible from postnatal days 8–10 onward. Around the age of weaning, which is usually 3–4 weeks, the anogenital distance in male pups is about 50% greater than that in females. In addition, the penis in male mice is fully developed by 18–28 d after birth. In adult male mice, the scrotum and testes are clearly visible.

In many genetic and developmental studies using newborn pups, it is necessary to determine pups' gender at a very early stage. In neonatal mice, gender is typically assessed by measuring the anogenital distance, which is greater in males than in females. This method, however, has proven to be difficult and not completely reliable. The anogenital distance of a newborn pup is best visualized by bending the lower back of the pup slightly backwards, which stretches the genital area. This is one of the weaknesses of the method; depending on the extent to which the lower back is bent, the anogenital distance may vary. This may result in an incorrect assessment of a pup's gender.

Furthermore, some litters contain pups of one gender only, and the lack of opportunity to compare between male and female pups can make gender determination more difficult, particularly for the inexperienced handler. In view of the above limitations, we propose an additional method for gender determination in newborn mice.

A method to 'spot' the gender in neonatal pigmented mice

In pigmented mice, an alternative and more reliable characteristic that can be used to determine the gender of a mouse pup is the presence of a pigmentation spot

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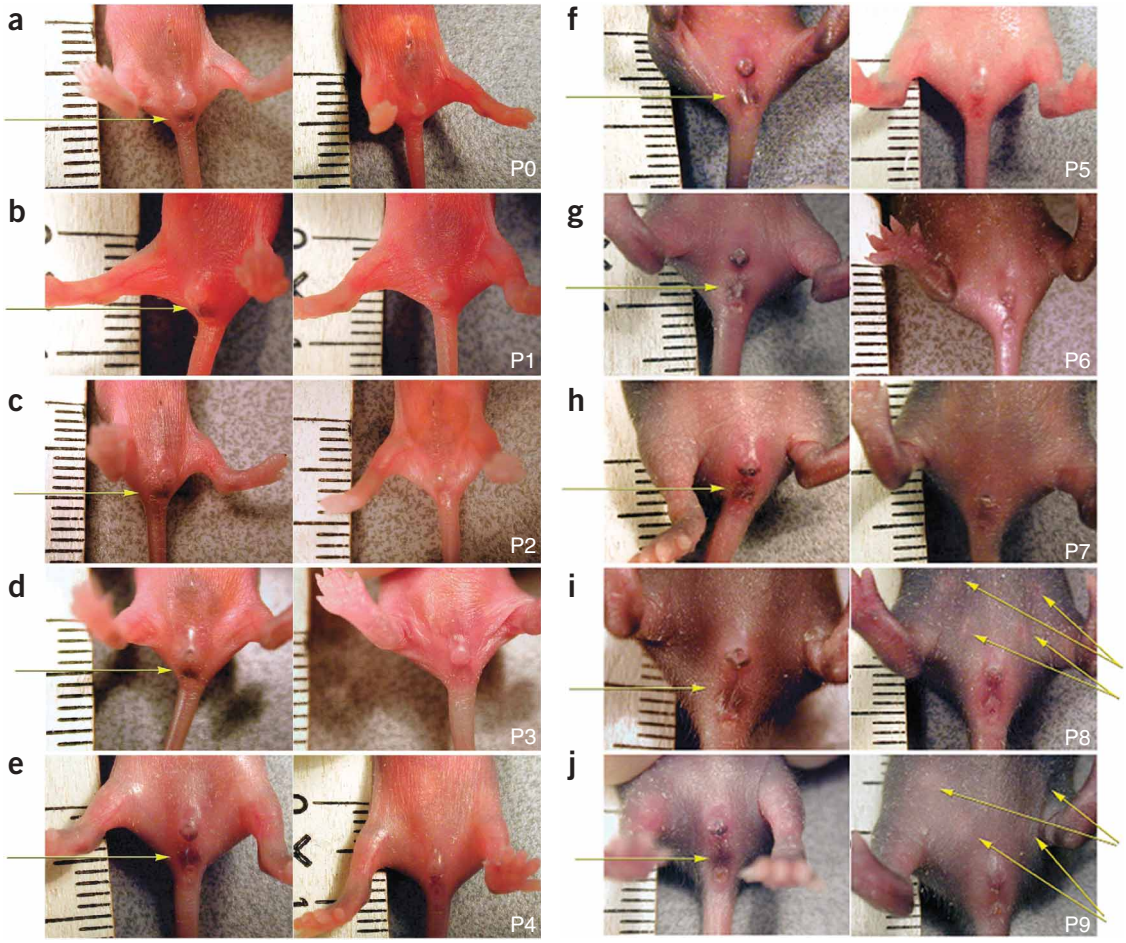

FIGURE 1 | Difference in anogenital pigmentation of neonatal male (left) and female (right) C57BL/6J mice from P0 to P9. In the male, the pigmented spot on the scrotum (arrow) is visible from P0 (**a**) onwards. In the female, the nipples are clearly visible as pink dots (arrows) from P8 (**i**) onwards.

on the perineum of the male mouse between the genital papilla and the anus. This area later develops into the scrotal sac. The presence of a pigment spot in male mice has been recognized previously as a marker of gender[1,2]. With the goal of providing a technical aid for gender assessment, we present here a photographic record of the development of this pigment spot in C57BL/6J mice (**Fig. 1**).

In male pigmented pups, pigmented cells on the scrotum are visible to the naked eye on the day of birth (postnatal day 0; P0), provided that the pups are clearly illuminated (**Fig. 1a**). Immediately after birth, these cells may be quite difficult to see (hence the requirement for good illumination), but within a few hours the intensity of the pigmentation increases substantially. By P1, the pigmented spot is clearly visible (**Fig. 1b**). There is no visible pigment spot in female mice (**Fig. 1**).

We and others[3] have observed that on gestation days 19–20 (just before birth), the scrotal pigmentation spot in male pups is not visible to the naked eye. Notably, the pigmentation spot consistently appears on the day of birth, irrespective of the duration of gestation (**Fig. 1**). A study using histological techniques has shown that the pigment spot begins to develop on day 16 after fertilization but is only visible microscopically at that stage[3]. The pigment spot is the result of a high density of melanocytes with longer, heavily pigmented dendrites in the dermis of the skin. These melanocytes become restricted to the dermis during embryonic development. In females, this concentration of pigment cells is much lower than in males.

Pigmentation in the scrotum is controlled by testosterone in several animal species, including the rat, golden hamster, ground squirrel, guinea pig and monkey[4]. In hypogonadal and castrated men[4] and in castrated Long-Evans rats[5], testosterone has been reported to induce pigmentation of the scrotum. The study in rats showed that increased pigmentation resulted from an increase in melatonin levels in the cell rather than an increase in cell number[5].

According to our observations, in lightly pigmented mouse strains such as the DBA/2J and C57BL/6J-Chr 4A/NaJ strains, the pigment spot is not visible either

TABLE 1 | Strains in which anogenital pigmentation was assessed

Strain	Fur color	Number of pups tested	Scrotal pigmentation present?
A/J	White	20	No
C57BL/6J-Chr 7A/NaJ	White	20	No
129X1/SvJ	White	15	No
C57BL/6J-Tyrc-2J/J	White	100	No
DBA/2J	Dilute brown agouti	15	No
C57BL/6J-Chr 4A/NaJ	Brown/gray	30	No
129S6/SvEvTac	Agouti	10	Yes
C57BL/6J	Black	600	Yes
C57BL/6J-Chr #A/NaJ (not including Chr 7A/NaJ and Chr 4A/NaJ)	Black	465	Yes

on the day of birth or a few days later. Not surprisingly, non-pigmented mice do not have a visible pigment spot on the scrotum (**Table 1**). In these strains, the gender of newborn mice can only be identified on the basis of the anogenital distance.

Comparison with measurement of anogenital distance

To evaluate the accuracy and efficiency of using the pigmentation of the scrotum as a marker of gender in newborn dark pigmented mice (the 'spot on' method), we compared this method with measurement of the anogenital distance. Five inexperienced animal handlers used both methods to determine the genders of pigmented pups at P0 (pigmented strains were C57BL/6J and C57BL/6J crossed with CBA/J). Experimenters had no experience handling rodents in general or neonates specifically. An experienced animal handler verified the genders of mice 2 weeks later. We recorded the number of correct scores and the amount of time it took to assess the gender of each mouse (**Table 2**). With anogenital distance measurement, experimenters

TABLE 2 | Comparison of the 'spot on' (SO) method with measurement of the anogenital distance (AD)

Experimenter	Strain	Number of pups in litter	Method	Correct scores (#)	Average time to score each pup (s)	Time to score litter (s)
A	C57BL/6J	5	AD	4	13	65
		5	SO	5	6.6	33
B	C57BL/6J	5	AD	4	12.4	62
		5	SO	5	7	35
C	C57BL/6J	5	AD	4	11.8	59
		5	SO	5	5	25
D	C57BL/6J × CBA/J	12	AD	9	6.25	75
		12	SO	11	4.6	55
E	C57BL/6J × CBA/J	8	AD	7	9	72
		8	SO	8	3.8	30

All experimenters were inexperienced animal handlers. Each experimenter determined the gender of newborn mice (at P0) from two separate litters of the same strain.

assessed gender incorrectly 20% of the time, whereas the error rate was much lower with the 'spot on' method (2.9%). Anogenital distance measurement was also less efficient; experimenters measuring anogenital distance took 9 s on average to determine the gender of each pup, compared with 5 s per pup with the 'spot on' method. For experienced animal handlers, there was no difference between the two methods in the accuracy or speed of gender recognition (0% error rate and an average of 5 s per pup for both methods). Notably, inexperienced handlers using the 'spot on' method determined the gender of pups as quickly as did experienced handlers.

Conclusions

We have shown that use of the scrotal pigment spot in pigmented mice is an easy, time-saving method to assess the gender of mice postnatally from P0. The method is based on a clearly visible qualitative developmental marker and, with an observed accuracy of nearly 100%, is much more reliable than the quantitative anogenital distance method. Use of the 'spot on' method in combination with measurement of the anogenital distance will lead to an improvement in the training of inexperienced handlers. Furthermore, for experiments in which investigators must distinguish between genders of pre-weaned mice[6], use of this method may prevent animal wastage caused by incorrect gender determination.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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