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Combining sperm plug genotyping and coat color chimerism predicts germline transmission

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Abstract

There has been a significant increase in the use of C57BL/6N-derived ES cells for the production of gene knockout mice. However, the potential for germline transmission (GLT) from chimeras on this genetic background has been observed to be highly variable. Using coat color as an indicator of somatic chimerism to infer the extent of chimeric contribution to the germ cell population, even highly agouti C57BL/6N-derived chimeras can fail to achieve GLT. We investigated the extent to which quantitative PCR genotyping for a marker gene expressed in gene targeted ES cells can be performed on DNA extracted from sperm present in copulatory plugs to determine the contribution of ES cells to the germ cells. We found that an objective assessment of sperm DNA from copulatory plugs combined with a subjective assessment of coat color chimerism can be used to accurately inform the selection of chimeras for breeding that are likely to achieve GLT. These results indicate that, compared to random selection of chimeras, including an analysis of copulatory plugs to set chimeras for breeding can help to reduce costs, minimize time, and facilitate research for projects requiring the production, selection, breeding, and testing of chimeras to generate gene-targeted mice.

Keywords

ES cells; C57BL/6N; Chimera; Germline transmission; Copulatory plug; Quantitative PCR; Gene knockout

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Introduction

Gene knockout (KO) technology via genetic manipulation of embryonic stem (ES) cells in mice has become the most effective way to specifically delete gene expression and enable the study of gene function. Several international projects, including Knockout Mouse Project (KOMP) and the European Conditional Mouse Mutagenesis (EUCOMM) Programme, have used gene KO technology to create a comprehensive library of gene-targeted ES cell lines for all genes (Collin et al., 2007; Skarnes et al., 2011). One of the important goals of these projects is to make available these ES cells to the research community for conversion into mice for phenotypic analysis (Allan et al., 2012).

In these projects, C57BL/6N ES cells have most commonly been used for gene targeting (Pettitt et al., 2009; Auerbach et al., 2000). Unlike ES cell lines on other genetic backgrounds (e.g., 129/Sv), germline-transmission (GLT) confirmed KO mice made using gene targeted C57BL/6N ES cells can be rapidly derived by mating chimeras with C57BL/6N wildtype mice, thus precluding repeated backcrossing to achieve a pure, inbred genetic background. The decrease in time to achieve an isogenic background of gene knockout mice equates to significant cost and time saving for investigators.

To realize the full scientific benefit and potential of these resources, it is important to attempt to maximize the efficiency of each step in the process of generating gene KO mice for study. For example, a critical bottleneck deserving of enhanced efficiency is achieving GLT of the targeted gene in progeny generated by breeding male chimeras to female wildtype mice. This bottleneck arises from the observation that chimeras derived by injection of C57BL/6N ES cells are observed to be less germline competent than chimeras derived from 129/Sv ES cells (Hansen et al. 2008). Without informed guidance based on evidence, researchers attempt to generate as many high percentage coat color chimeras as possible and then randomly select all or at least the high-percentage males for GLT testing. This protocol leads to increases in per diem, mouse, and genotyping costs, prolongation of breeding, and delays in conducting research experiments on confirmed KO mice. Even when successful, GLT testing can require 5–6 months or more to produce a litter with confirmed ES cell-derived progeny. An additional complication of using C57BL/6 ES cell lines is their tendency toward aneuploidy, which limits the ability to produce viable chimeras suitable for breeding to achieve GLT (Hughes et al., 2007). These factors make selection of germline competent C57BL/6N ES cell derived chimeras for breeding to test GLT a challenging and complicated process, taxing precious time and limited resources.

To overcome this hurdle, somatic chimerism has been assessed by subjective visual estimation of coat color to infer the proportional contribution of ES cells to the germ cell population as the primary criteria for the selection of chimeras for breeding to achieve GLT. However, unlike for chimeras derived from 129/Sv ES cells (Hansen et al, 2008), the GLT potential of chimeras derived from C57BL/6N ES cells does not correlate well with coat color chimerism. Alternatively, copulatory plugs from chimeric males can be harvested and used for quantitative PCR to genotype the proportion of ES cell derived sperm. In a recent study, real-time PCR was used to successfully detect ES cell derived genetic markers (neomycin resistance gene, *neo*) in sperm DNA extracted from copulatory plugs (Wilson & Sheardown, 2011). However, because this study reported on only a few C57BL/6 derived chimeras, there was insufficient data to enable a reproducible correlation between the strength of the mutation signal (i.e. *neo*) with the rate of GLT. Nonetheless, it is rational to hypothesize that quantitative determination of germ cell chimerism should be a better predictor of GLT than qualitative determination of somatic cell chimerism by coat color. Another benefit is that sperm DNA from copulatory plugs can be obtained without sacrificing the mouse.

Therefore, in this report, we present data correlating a subjective assessment of somatic chimerism by coat color with an objective measurement of germ cell chimerism in copulatory plugs and with the GLT potential for individual chimeras derived from C57BL/6N ES cells. Using quantitative PCR to detect a LacZ gene marker expressed in gene targeted ES cells, the goals of this study were to determine if there is sufficient evidence to support the hypothesis that DNA extracted from copulatory plugs 1) is a non-invasive and reliable source of sperm for genotypic analysis, 2) provides a means to directly correlate *LacZ* signal strength in copulatory plug DNA to coat color chimerism, and 3) exhibits a direct correlation with the GLT potential of a chimera. We also wanted to infer whether analysis of copulatory plugs could be used to predict the likelihood for success of GLT testing. We found that genotyping sperm DNA from copulatory plugs can inform the selection of germline competent chimeras for breeding to achieve GLT. These results indicate that the analysis of copulatory plugs can help to reduce costs, minimize time, and facilitate research for projects requiring the production, selection, breeding, and testing of chimeras to generate gene-targeted mice.

Materials and Methods

ES cell lines and generation of chimeras

In the KOMP, several different C57BL/6N ES cell lines and sublines were used for gene targeting: JM8, JM8A3.N1, JM8A1.N3, JM8.N4, JM8.F6 (Pettitt et al., 2008) and VGB6 (Poueymirou et al., 2006). Using established protocols (www.komp.org), homologously-recombined ES cell clones were cultured to sub-confluent density and single cell suspensions prepared by application of Accutase (Invitrogen, Carlsbad, CA, USA) on the day of microinjection into either blastocysts or 8-cell stage morulae. Approximately 17–18 clonal ES cells were injected into 30–34 blastocysts or morulae harvested from 4 week old BALB/c females (mated with BALB/c males) or C57BL/6N females (mated with B6D2F1 males). Injected morulae were cultured overnight in KSOM (Millipore, Billerica, MA, USA) at 37°C and 5% CO₂. The following day, both injected blastocysts and blastocysts developed from injected morulae were transferred into the uterus of pseudopregnant CD1 females and allowed to litter. At 10–12 days after birth, the number, sex, and extent of coat color chimerism in offspring was determined and recorded.

Chimeras breeding and copulatory plug collection

Following weaning and reaching sexual maturity at approximately 6–7 weeks of age, all surviving male chimeras were set with 4 weeks old CD-1 females at a 1:1 ratio for 1 week. All CD-1 females were visually inspected daily in the morning for copulatory plugs. When a plug was detected, the female was removed from the breeding box with the chimera and euthanatized by CO₂ narcosis and cervical dislocation. Plugs were collected and stored at –20°C until DNA extraction. Once removed from the chimera, the CD-1 female was then replaced with 2 C57BL/6N females for GLT testing for 45 days, or until 45 wild type pups were obtained. A chimera that produced heterozygous mutant pups was recorded as a successful GLT. In contrast, a chimera that failed to produce heterozygous offspring within 45 days or produced only wild type progeny was recorded as an unsuccessful GLT.

DNA extraction and quantitative PCR

Sperm plug DNA was extracted using Qiagen DNeasy Blood & Tissue kit following manufacturer's protocol with an extended proteinase K incubation of 20 hours. DNA was then assayed using Taqman® quantitative PCR via the multiplexed relative cycle threshold method. Briefly, 2µl of each DNA sample was tested in a total reaction volume of 10µl in duplicate utilizing Qiagen QuantiTect PCR kit with ROX chemistry. 384 well plates were processed on an AB7900HT system with the following cycles: 95°C for 15 minutes and then

40 cycles of 95°C for 30 seconds, 60°C for 1 minute. Primers and probe for detection of mutant sequence (*LacZ*) in the final concentration of each reaction were forward primer ATCAGGATATGTGGCGGATGA (0.84μM), reverse primer TGATTTGTGTAGTCGG TTTATGCA (0.84μM) and probe 6FAM-CGGCATTTCCTG-MGB/NFQ (0.28μM). Primers/probe for endogenous reference sequences were in the final concentration of each reaction: forward primer GTCATCAAGTGAGAAAGACATCCT (0.84μM), reverse primer CATCATGAATTTTGATAAGCCCATT (0.84μM), and probe VIC-CTCCTGGCTGCCTG-MGB/NFQ (0.28μM). Each sperm DNA sample with its averaged Ct was compared against the averaged Ct of heterozygous mutant animal DNA (Ct_{Het}). Percent mutation of sperm cells was calculated as $\% = 2^{-(Ct_{Sperm} - Ct_{Het})}$ in which ratio is expressed as a decimal. ES cell derived *LacZ* signal of DNA samples are shown in graphs were expressed as a ratio of *LacZ* signal in the sperm DNA normalized to *LacZ* signal in mutant heterozygous DNA.

Result and Discussion

LacZ signal obtained from sperm DNA in copulatory plugs

By quantitative PCR (qPCR) of DNA extracted from copulatory plugs, the proportion of ES cell derived sperm was determined by the presence of *LacZ* signal. Of 275 copulatory plug samples obtained from 262 chimeras generated after injection of 87 ES cell clones for 87 unique genes, 216 (79%) had detectable *LacZ* signal, indicating that qPCR is able to detect *LacZ* in DNA extracted from sperm and to confirm the presence of ES-cell derived sperm in copulatory plugs. The remaining 21% of samples had no measurable *LacZ* signal. This result likely reflected the presence of little to no ES cell-derived sperm in the copulatory plug, either because the ES cells did not contribute to ontogeny of the germline and/or the sperm derived from ES cells were slower to develop to mature spermatogonia compared with those from the wild type host (Wilson et al. 2011). Although sperm is the primary component of a copulatory (“sperm”) plug, other components also include seminal fluid, mucus, and female vaginal epithelial and blood cells. However, the presence of a copulatory plug does not always result in pregnancy, which can fail due to many factors such as poor yield of sperm or lack of ovulation (Deb et al. 2006). In general, 80–90% of females with plugs will deliver a litter, implying that the presence of sperm in the copulatory plug is sufficient to fertilize the egg that then implants into the uterus. Therefore, our results confirm our hypothesis that copulatory plugs are a reliable source of DNA for qPCR detection of ES cell-derived sperm in chimeras. Additionally, our results also indicate that the ES cell contribution to germ cells can be determined non-invasively in live animals without the need to cull the male chimera (Fig. 1).

LacZ signal strength in related to coat color chimerism

When chimeras reached 10–12 days of age, the extent of somatic chimerism was determined by visual examination of coat color (Fig. 2). By comparing the qualitative proportion of ES cell derived coat color with the *LacZ* signal strength of the copulatory plug, we hypothesized that somatic chimerism would correlate directly with germ cell chimerism. However, this was not the case (Fig. 3) (R^2 of linear regression was only 0.054). Instead, we found that *LacZ* signal strength varied considerably, from none to strong (i.e. 0–1), in mice with high (50%) coat color chimerism. However, for mice with low (<50%) coat color chimerism, *LacZ* signal strength was consistently in the low range (0–0.1), with very few samples showing stronger (>0.1) signal strength. The weak correlation between high coat color chimerism and *LacZ* signal strength suggests that highly chimeric male mice derived from C57BL/6N ES cells might not all have high GLT potential. This observation is consistent with the results of a previous study (Hansen et al., 2008) in which mice derived from C57BL/6N ES cells derived were likely to have GLT potential similar to mice derived from 129/Sv

ES cells only when the proportion of coat color chimerism was >90%. Our study helps to explain this previous observation, in that the copulatory plugs produced by the higher percentage (50–90%) coat color chimeras contained few to no ES cell derived sperm as determined by the *LacZ* signal strength in the copulatory plug.

Correlation of copulatory plug *LacZ* signal strength with rate of GLT

In chimeras that successfully achieved GLT, we analyzed the range of *LacZ* signal strength in their copulatory plug. *LacZ* signal strength varied between 0.02 and 1, and 91% of samples showed ≥ 0.14 . Also, coat color chimerism of mice that achieved GLT showed weak correlation with the copulatory plug *LacZ* signal strength (Fig. 4). Logically, the strength of the copulatory plug *LacZ* signal should be directly dependent on the quantity of ES cell derived sperm in the copulatory plug. However, we observed that the *LacZ* signal varied widely in different copulatory plugs from the same male, indicating that the amount of ES cell derived sperm can similarly vary between ejaculations (data not shown). This observation could be explained by results suggesting that ES cell derived sperm in chimeras mature at different rates compared with those derived from the wild-type host (Wilson et al. 2011).

In chimeras which failed germline transmission (no progeny within 45 days of breeding or production of only wild type pups), the copulatory plug *LacZ* signal strength varied from 0–0.64. However, the majority (91%) of plugs had a significantly low (<0.14) *LacZ* signal strength. Similarly, coat color chimerism of mice that failed GLT correlated weakly with the copulatory plug *LacZ* signal strength (Fig. 5). Therefore, except for extremely high (>90%) coat color chimerism, somatic chimerism does not necessarily predict germline transmission. However, several high percent coat color chimeras producing copulatory plugs with weak *LacZ* signal strength failed GLT, indicating low numbers of ES cell-derived sperm. Instead, we found that GLT of chimeras could be better predicted when coat color chimerism *and* copulatory plug *LacZ* signal strength were analyzed *together* than when analyzed independently (Fig. 6). Chimeras with copulatory plug *LacZ* signal strength greater than 0.14 were more likely to go germline. This is because 91% of chimeras that passed GLT testing had a copulatory plug with a *LacZ* signal strength ≥ 0.14 , while 91% of chimeras that failed GLT testing had a copulatory plug with a *LacZ* signal strength <0.14 . In addition, germline competent mice that produced copulatory plugs exhibited at least >50% coat color chimerism. A larger percentage of germline-competent mice passed GLT testing when their coat color chimerism was >70%. Our data are consistent with observations in a previous study (Hensen et al., 2008) in which C57BL/6N derived chimeras with >70% coat color chimerism had double or more the rate of GLT compared to 50% coat color chimeras.

Conclusion

Direct assessment of somatic chimerism by coat color is a common subjective means by which to indirectly infer germ cell chimerism to select mice for GLT testing. In the majority of samples examined using qPCR, we were able to consistently genotype for *LacZ* in DNA extracted from copulatory plugs of chimeric males to quantify the proportion of ES cell derived sperm. Our results indicate that the copulatory plug is a reliable source of ES cell derived sperm DNA. When comparing the copulatory plug *LacZ* signal strength to GLT potential of individual chimeras, we found that a mouse with a copulatory plug *LacZ* signal strength <0.14 was unlikely to achieve GLT. On the other hand, when analyzing copulatory plug *LacZ* signal strength and coat color chimerism *together*, we found that most (>90%) chimeras exhibiting 50% or greater coat color chimerism and a copulatory plug *LacZ* signal strength ≥ 0.14 in *LacZ* were likely to achieve GLT.

In general, chimeras generated from C57BL/6N-derived ES cells are less likely to achieve GLT compared to comparable chimeras generated from ES cells derived from 129/Sv, even at high levels of somatic chimerism as determined by coat color. Because determination of the extent to which ES cells contribute to color coat is highly subjective and thus can vary between individuals, we recommend investigators apply a more objective assessment. Our study indicates that quantitative measurement of the signal strength of a reporter gene (e.g. *LacZ* or *neo*) in DNA extracted from the sperm present in copulatory plugs can be used as an objective criteria when selecting chimeras most likely to achieve GLT. This is especially useful when vivarium space and resources are limited. When possible, for mice derived by gene targeting in C57BL/6N ES cell lines with relatively low rates of GLT, we suggest producing chimeras from 3–4 individual clones per gene for GLT testing (Hansen et al, 2008 and Pettitt et al, 2009). Chimeras with the highest likelihood of achieving GLT can then be selected for breeding using copulatory plug testing. Although copulatory plug testing requires an additional week of mating, this step can assist one to select from all chimeras produced those with the highest GLT potential, which will contribute to saving costs and time compared to GLT testing of all chimeras. Our results indicate that assessing coat color chimerism together with copulatory plug testing to select chimeras to breed for GLT can lead to a faster and more efficient production of gene knockout mice for study.

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Normalized *LacZ* signals from copulatory plugs of germline chimeras **Fig. 1.**

LacZ signal strength in the germline chimeras. Bars represent the normalized *LacZ* mutant cells detected. All 45 germline chimeras were derived from C57B/6N ES cells.

Fig. 2.
Examples of coat color chimerism in percentage. Only C57BL/6N ES cell derived chimeras generated by microinjection of ES cells to BALB/c blastocysts are shown.

Fig. 3.

Correlation between chimera coat color chimerism and *LacZ* signal from copulatory plugs. Dots represent the normalized *LacZ* signal to the coat color chimerism of individual chimera. Total number of chimeras was 263. Number of chimera with coat color chimerism (10–50%) was 53, Number of chimera with coat color chimerism (50%–100%) was 210. R^2 represent R-squared value of the linear regression.

Fig. 4. Correlation between *LacZ* signal strength and coat color chimerism of germline chimeras. Dots represent the normalized *LacZ* signal to the coat color chimerism of individual chimera. All 45 germline chimeras were derived from C57B/6N ES cells. R^2 represent R-squared value of the linear regression.

Fig. 5.

Correlation between *LacZ* signal strength and coat color chimerism of germline failed chimeras. Dots represent the normalized *LacZ* signal to the coat color chimerism of individual chimera, red dots are chimeras had 0.14 in *LacZ* signal strength. All 65 germline failed chimeras were derived from C57B/6N ES cells. R^2 represent R-squared value of the linear regression.

Fig. 6.

LacZ signal strength and coat color chimerism with overlapping of germline passed (Fig. 4) and germline failed chimera (Fig. 5). Green or blue dots represent the normalized *LacZ* signal to coat color chimerism of germline passed or failed chimera respectively. The red dash line is the cut-off for 50% of coat color chimerism and purple dash line is the cut-off for the normalized *LacZ* signal 0.14.