Published in final edited form as:
Brain Behav Immun. 2009 March ; 23(3): 318–324. doi:10.1016/j.bbi.2008.09.001.

Cautionary Insights on Knockout Mouse Studies: The Gene or Not the Gene?

Amy F. Eisener-Dorman[1,2], **David A. Lawrence**[1,2], and **Valerie J. Bolivar**[1,2]

[1] Wadsworth Center, 120 New Scotland Avenue, Albany, New York 12208, USA

[2] School of Public Health, Department of Biomedical Sciences, State University of New York at Albany, Albany, New York, 12201, USA

Abstract

Gene modification technologies play a vital role in the study of biological systems and pathways. Although there is widespread and beneficial use of genetic mouse models, potential shortcomings of gene targeting technology exist, and are not always taken into consideration. Oversights associated with the technology can lead to misinterpretation of results; for example, ablation of a gene of interest can appear to cause an observed phenotype when, in fact, residual embryonic stem cell-derived genetic material in the genetic background or in the area immediately surrounding the ablated gene is actually responsible. The purpose of this review is to remind researchers, regardless of scientific discipline, that the background genetics of a knockout strain can have a profound influence on any observed phenotype. It is important that this issue be appropriately addressed during data collection and interpretation.

1. Introduction

Gene targeting is critical to advances in many fields of research, ranging from immunology to neuroscience to genetics. This fact is evident from the prominence of knockout mouse studies in the literature (e.g., a search of “knockout mice” yields more than 50,000 hits in PubMed). There is no denying the fact that the availability of genetic mouse models has revolutionized biomedical research; further, recently established alterations to classical gene modification techniques have made possible the generation of conditional, inducible, and even multiple-gene knockout mouse mutants. Gene-targeting technologies deliver a more informative look into the *in vivo* functions and endogenous expression patterns of individual genes. Thus, the promise of this technology has spurred the generation of a plethora of knockout mice with ablated genes that are involved in diverse biological pathways and systems.

It is tempting to conclude that the phenotypes observed in any given knockout mouse are completely due to the ablation of the gene of interest, and researchers often do make this assumption. In many cases, they are correct. However, as we will discuss in this review, traditional knockout technology has commonly overlooked limitations. One of these limitations is the presence of regions of genetic variability (“passenger” or “flanking” genes) that are transported with the “knocked out” gene onto the selected genetic background. We will highlight how these genetic regions can have a critical impact on the interpretation of phenotypic data, as illustrated in a recent behavioral study of interleukin-10 knockout mice

(Rodrigues de Ledesma et al., 2006). In addition, we will present various approaches that have been designed to abrogate flanking gene and genetic background complications and discuss modifications to conventional knockout methods that can circumvent the issue of flanking genes entirely.

2. Generation of a Knockout Mouse: Strengths and Limitations

Researchers must be aware of the basic principles of current gene modification technology before the inherent complications arising from flanking and/or background genes can be fully appreciated. The basic premise behind the development of a knockout mouse is to replace the normal functioning gene with one that is nonfunctional (i.e., a null mutation). The generation of a knockout mouse has been described in detail previously (Nagy et al., 2003; Papaioannou and Behringer, 2004) and will only be briefly reviewed here. The first step of the process is to introduce a null mutation of the gene of interest into a critical exonic sequence. This sequence is subsequently inserted into the targeting vector. In addition to the disrupted gene, another gene is included between the homologous regions. Generally, this is an antibiotic resistance gene, which will allow selection of cells in which the vector has integrated. The mutation-carrying targeting vector is introduced into embryonic stem (ES) cells (Fig. 1), where it integrates into the genome via homologous recombination. Antibiotic resistant ES cells (those that have incorporated the mutagenized DNA construct) are then used for injection into blastocysts, which are in turn transferred into pseudopregnant females for production of mutant mice. Importantly, several 129-derived cell lines, originating from the Parental and Steel substrains (described in Section 3), remain the most commonly used ES cells for this process because they are easily derived as germline competent lines, amenable to genetic manipulation, and readily expanded (Simpson et al., 1997).

The next stage of the process is the generation of gene-targeted, or knockout, mice. ES cell clones carrying the null mutation are implanted into blastocysts, usually C57BL/6 (B6) in origin, although blastocysts from other strains are used in some cases. The blastocysts are implanted into pseudopregnant females, and the combination of 129 and B6 cells results in chimeric mice that are selected for germline transmission. Finally, these chimeras are selectively bred so as to maintain the ablated gene region on the preferred genetic background. A coisogenic knockout strain is generated by crossing chimeras to mice of the same 129 substrain that was used to generate the knockout (Fig. 1, Approach #1). It is reasonable to assume that the resulting mice are essentially a specific 129 substrain with a single-gene ablation. Although generation of a coisogenic knockout strain avoids genetics-based issues, 129 substrains are notoriously poor breeders (Seong et al., 2004) that display undesirable traits that complicate phenotypic characterization. B6 is the most widely used background strain for knockout mice, largely due to the fact that they breed well and exhibit fewer physiological and behavioral abnormalities than do the 129 substrains. Knockout mice are often extensively backcrossed to B6 to generate knockout/congenic strains with approximately 99.9% B6 background (after at least 10 generations of backcrossing) (Fig. 1, Approach #2). Unfortunately, researchers often incorrectly assume that the process results in a B6 mouse with a single-gene ablation, but that is simply not the case.

The primary issue with gene targeting technology is that 129 ES cell-derived genetic material will remain in the genome to some extent, despite the best efforts of researchers to breed it out. The potential for data misinterpretation due to either “flanking gene effects” or “genetic background effects” has been particularly well chronicled within the field of behavioral genetics (Bolivar et al., 2001a; Crusio, 2004; Gerlai, 1996; Wolfer et al., 2002). Fortunately, researchers in diverse scientific fields are becoming increasingly aware of the broader implications of this issue (Ridgway et al., 2007). The term “flanking region” refers to the fact that the ablated gene is flanked by indeterminate regions of 129-derived genetic material. These

regions potentially contain hundreds of genes, any of which can produce an observable phenotype. The close proximity of this flanking region to the ablated gene of interest results in a high degree of genetic linkage, thus genetic recombination through extensive backcrossing cannot facilitate complete replacement of the flanking regions with B6 genetic material.

That is not to say that backcrossing to create knockout/congenics lacks genetic benefits. In fact, such an approach does narrow the flanking region considerably, thus lessening – but not eliminating – the potential phenotypic impact of this region through a reduction in the number of 129-derived genes. In addition, generation of a knockout/congenic strain allows the effects of the gene of interest to be studied on a primarily B6 genetic background, thereby minimizing the phenotypic complications intrinsic to the study and maintenance of a knockout on a 129 genetic background (i.e., a coisogenic knockout strain). The term “genetic background effect” refers to the fact that, in spite of extensive backcrossing, knockout/congenics do not have a 100% B6 background. These strains still harbor discrete regions of residual 129-derived material that may act in isolation or produce epistatic interactions with the ablated gene of interest. Hence, such unlinked loci residing at unidentified locations throughout the B6 genome can be solely or partially responsible for observed phenotypes.

3. Genetic Background and Flanking Genes: Why They Matter

Inbred strains, classically defined by brother/sister mating for at least 20 generations (Silver, 1995), exhibit vast genetic and phenotypic variability. As stated previously, the mouse strains most often used in the generation of knockout mice are 129 and B6. Because these two strains have been shown to differ in many regards, genetic analysis of knockout strains is just as essential as their phenotype characterization. 129-derived ES cells are the best-developed and most reliable ES cell type for gene modification purposes; however, 129 substrains provide an unfavorable genetic background, as most substrains are characterized by poor reproductive performance, neuroanatomical (Wahlsten et al., 1992), and behavioral abnormalities. More specifically, 129 substrains exhibit responses that differ from B6 in a wide range of behavioral parameters, including grooming, activity and habituation in the open field, motor ability and motor learning, learning and memory, cued and contextual fear conditioning, depression-like behaviors, anxiety, and drug-induced behaviors (Bolivar et al., 2000; Bolivar et al., 2001b; Bothe et al., 2004; Crawley et al., 1997; Ducottet and Belzung, 2005; Kalueff and Tuohimaa, 2004; McFadyen et al., 2003; van Bogaert et al., 2006).

Furthermore, many differences exist among the various 129 substrains. Substantial genetic variability among substrains has been linked to several instances of genetic contamination (Simpson et al., 1997; Threadgill et al., 1997). As a result, the 129 substrains have been classified into several lineages: Parental (P; white-bellied, pink-eyed, light chinchilla), Steel (S; white-bellied agouti), Ter (T; white-bellied chinchilla), and genetically contaminated (X; white-bellied chinchilla) (http://www.jax.org). This extensive genetic variability results in 129 substrain differences in physiology (Sechler et al., 1997) and behavior (Cook et al., 2002; Montkowski et al., 1997; Paylor and Crawley, 1997). The Mouse Phenome Database (http://www.jax.org/phenome), maintained by The Jackson Laboratory, is a valuable source of inbred and outbred mouse phenotypic data acquired by members of the international scientific community as part of the Mouse Phenome Project. Information provided by this resource includes single-nucleotide polymorphisms (SNPs), reported quantitative trait loci (QTLs), and a wide range of physiological and behavioral phenotypes associated with many inbred mouse strains. Therefore, it is imperative to identify the 129 substrain used as the ES cell source for generation of the knockout strain and to use the same 129 substrain for further phenotypic characterization. Such well-documented variability among inbred strains and 129 substrains must be taken into account when designing experiments and interpreting data.

4. What's a Scientist To Do?

Despite the limitations to knockout mouse technology that have been presented herein, there are several methods that can be used to simplify data interpretation. Such methods either modify the conventional knockout technology or aid in the identification of the genetic origin of a phenotype. While some of these approaches involve an understanding of the underlying genetics, others employ more basic techniques. Regardless of the method used, the question is essentially the same: is the ablated gene under study truly responsible for the observed phenotype?

4.1 Use of knockout/congenics

Knockout mouse strains are commonly backcrossed to B6, an inbred strain that has been well characterized. This effectively minimizes the influence of the 129-derived genome, while simultaneously endowing the knockout strain with the benefits of a B6 background. As described earlier, many knockout mouse strains are eventually backcrossed for 10 or more generations to B6, thereby generating a congenic B6 background (Fig. 1, Approach #2). Such knockout/congenic mouse strains have been successfully used in genetic, immunological, and behavioral studies. While this approach certainly abrogates some of the issues inherent in using the 129 strain for gene-targeting studies, not all 129-derived genetic material is replaced with B6. The genetic region immediately surrounding the ablated gene is unlikely to undergo recombination with the B6 genome without resulting in the simultaneous loss of the gene ablation simply due to their close proximity. Thus these regions remain inextricably linked to the knockout locus.

Further, it is not uncommon to discover unlinked 129 loci on a congenic B6 background. In the process of backcrossing to develop a congenic strain, recombination is generally random throughout the genome. Thus, not every locus will be replaced by B6. These genetic background effects are particularly difficult to deal with since 129-derived loci can reside virtually anywhere in the genome. Perhaps the easiest and least expensive way to broadly scan the genetic background for regions of variability is by whole-genome genotyping using the thousands of known single-nucleotide polymorphisms (SNPs) (Fig. 1, Approach #3). In a behavioral study of interleukin-7 receptor knockout mice (B6.129S7$^{Il7r-/-}$), an open field phenotype was originally attributed to ablation of the gene; however, later findings, combined with whole-genome genotyping data, indicated that variability in the genetic background is responsible for the behavioral phenotype (Eisener-Dorman and Bolivar, unpublished data). Unfortunately, these 129-derived genes do not always occur in genomic regions for which SNPs are available. Although the genotyping approach is unlikely to identify every 129-derived locus, it is a reasonable starting point that may help identify regions of variability containing one or more genes that influence the observed phenotype.

4.2 Use of existing knockout strains to map phenotypic traits

Bolivar and colleagues (2001a) devised a breeding scheme using classical mouse genetics that can distinguish between the effects of the knocked out gene and effects of the 129-derived genetic material that flanks the ablated gene (Fig. 1, Approach #4). For optimal accuracy in this method, the knockout mouse strain of interest must first be generated as a knockout/congenic strain to minimize the existence of 129-derived genetic regions. The breeding scheme has three basic stages (Fig 1). The first stage of the breeding scheme establishes whether or not the homozygous knockout/congenic strain is phenotypically different from B6. If there is a difference between the knockout/congenic and B6, it may be due to the ablated gene or 129-derived genes located in the flanking region or the rest of the genetic background. In the second stage, the mode of genetic transmission is determined by breeding the homozygous knockout/congenic to B6 to generate F1 progeny that are heterozygous for both the ablated and 129-

derived alleles flanking region and then comparing F1 and B6 mice for the phenotype of interest. These findings determine which of the two possible approaches is necessary for the third stage of the breeding scheme.

If the heterozygous knockout/congenic F1 progeny are phenotypically similar to B6, the phenotype is classified as recessive. In this case, the genetic origin of the phenotype can be determined by crossing both B6 and the homozygous knockout/congenic mice to the 129 substrain from which the ES cells were derived (Stage 3B). Thus, if the recessive phenotype is due to 129-derived flanking genes, the (129 × B6) F1 progeny will exhibit a phenotype different from that of the (129 × B6-KO$^{-/-}$) F1 progeny. Given the 129 substrain differences described earlier, the validity of the breeding scheme can be compromised if the 129-based crosses are generated using a 129 substrain other than the one that was used to generate the knockout. Unfortunately, live mouse repositories are not maintained for all 129 substrains used in the derivation of ES cell lines. A related 129 substrain can be substituted for the ES cell-originating substrain, although the researcher should recognize that differences may persist, even between substrains within a common lineage.

If, on the other hand, the heterozygous F1 progeny differ phenotypically from B6, it is classified as a dominant or additive effect. In this instance, the knockout/congenic strain must be backcrossed extensively to B6 to generate recombinant mice that are then phenotypically screened (Stage 3A). This process, although time-consuming, effectively maps and validates the gene(s) responsible for the phenotype of interest. Unfortunately, this level of breeding and genetic analysis is not practical for many researchers. Other approaches may be more appropriate under these circumstances.

Paradoxically, the same residual 129 genetic regions that complicate phenotypic assessment of the ablated gene in conventionally generated knockout mice can also be used to map quantitative trait loci (QTL). A QTL is a genetic region at which allelic variation is associated with phenotypic variation. Quantitative trait loci have been identified in a wide variety of biological parameters, including growth (height, weight), behavioral traits (anxiety, sociability, aggression), and disease pathology (susceptibility, progression, severity). The IL-10 knockout study (Rodrigues de Ledesma et al., 2006), to be described below, provides an example of how a phenotype could have been erroneously attributed to the ablated gene, and demonstrates how knockout/congenics may be strategically utilized for QTL mapping.

4.3 Use of C57BL/6-derived embryonic stem cells

One way to eliminate the problem of the flanking region is to use ES cells derived from B6 rather than 129 mice (Fig. 1, Approach #5). To date this approach has not been widely used. Although the first B6 ES cell line was derived as early as 1991, few lines have been successfully used in the past decade. This is partially due to technological problems with B6 ES cells (Seong et al., 2004). However, with the poor breeding performance of some of the 129 substrains, it appears that B6 ES cells, which provide a pure B6 background for a gene of interest, are worth the technological challenges (Seong et al., 2004). In 2006, the NIH funded what is known as the Knockout Mouse Project (KOMP), with the immense goal of creating and providing public access to a repository of B6 ES cells that contain a null mutation for each gene in the mouse genome (Collins et al., 2007). The primary objective of this project is to generate knockout mice using genetically engineered B6 ES cells, thus completely eradicating concerns regarding contamination due to genetic background or flanking genes of 129 origin. Although the project is not yet complete, the resulting ES cell lines hold tremendous promise for future knockout mouse research.

4.4 Phenotype Rescue

The approaches described above involve either modifications to the gene-targeting technology itself or modifications of the generation and screening of informative crosses. However, one of the most straightforward and conclusive ways to determine if ablation of a gene is responsible for a phenotype is to rescue the knockout phenotype by *in vivo* complementation (Fig. 1, Approach #6). While rescue can be accomplished by any of a variety of methods, this review will focus only on some of the most common.

Bacterial artificial chromosome (BAC) transgenesis can be used to rescue a knockout strain phenotype, via introduction of a wild-type copy of the gene of interest back into the knockout mice. The process entails the design of a BAC construct that contains the wild-type gene of interest, microinjection of the resulting construct into embryos, and, finally, implantation of the embryos into pseudopregnant females. Transgenic mice are identified by genotyping for the BAC transgene, and these animals are crossed to knockout mice to generate mice that carry the BAC transgene on the knockout background. This approach is often used for candidate gene validation. For instance, this method successfully validated the contribution of the gene *Ltap* to the loop-tail mouse mutant phenotype (Kibar et al., 2003), which is characterized by a looped tail, enlarged lateral ventricles in the brain in heterozygotes, and severe neural tube defects in homozygotes. One of the primary advantages of BAC transgenesis over other *in vivo* complementation approaches is that the gene is present during development and into adulthood. Thus, the transgene should theoretically be expressed in the same manner as would be the endogenous gene itself, although it is important to note that transgene expression levels directly correlate with transgene copy number (Chandler et al., 2007).

Compared to the conventional knockout system or BAC transgenesis, conditional and inducible gene knockout technology confers greater *in vivo* control over gene expression. Conditional knockouts have tissue-specific inactivation of the gene of interest, rather than having direct effects on all biological systems within the organism. This effect is mediated by means of a recombinase, which is an enzyme that deletes the DNA fragment located between the two recombinase-specific sites. A mouse bearing the recombinase-specific sites is bred with a mouse expressing the recombinase. The tissue-specific expression of the recombinase allows the inactivation of the gene of interest to occur only in the tissue where the recombinase is expressed. An inducible knockout enables the researcher to activate or inactive the gene of interest at specific developmental timepoints. This is generally accomplished using antibiotic-controlled transcriptional activation, which is distinct from a conditional knockout. Thus, gene expression can be reversibly induced in the presence or absence of antibiotics such as tetracycline. If the observed phenotype changed, it is likely due to the gene of interest. Each of these knockout systems offers an opportunity to examine gene function at multiple timepoints and in various tissues, thereby benefiting candidate gene validation efforts.

Functional complementation can also be achieved at the protein level. Genes encode proteins or small molecules that can be exogenously administered, *in vivo* or *in vitro*, at almost any desired timepoint. For instance, many cytokines are readily administered to cytokine knockout mice in order to establish whether the knockout phenotype is, in fact, due to a deficiency of a particular cytokine. This approach can be quite effective in confirming the involvement of a specific gene in the corresponding knockout phenotype. However, the accuracy of the information yielded by this approach is dependent upon *a priori* knowledge of endogenous gene expression levels, tissue localization, and temporal expression patterns. Another limitation is that the wild-type protein expressed by the gene of interest can influence embryonic development. Thus, functional characterization at later timepoints (postnatal through adulthood) would be further complicated by any pre-existing condition(s) resulting from gene expression deficiency during development.

An alternative to phenotypic rescue of a knockout may be applicable, depending on the gene of interest. Many endogenous small molecules, e.g., receptor antagonists, antibodies, or binding proteins, have been identified as having the capacity to interfere with or completely block wild-type protein function in a tissue-, time-, and/or protein-specific manner. Exogenous administration of a small molecule that can block the effects specific to the protein of interest (e.g., IL-18 binding protein and IL-18) in a group of wild-type mice would provide a functionally deficient model analogous to a gene knockout strain on the same background. This approach may not be feasible if no such molecule is available for the protein expressed by a given gene of interest. However, when it is applicable, this method can complement knockout strain analyses.

5. Getting to Know Your Knockout: Characterization of an Interleukin-10 Knockout

5.1 Immunological perspectives on the functional role(s) of interleukin-10

Based on the potential influence of flanking genes, as discussed above, results from the use of knockout mice must be conservatively interpreted. For example, conclusions from IL-10 knockout mouse studies should be restricted to the known functions of the cytokine, unless the results can be confirmed with the use of mice lacking the IL-10 receptor and/or with the use of antibodies known to block IL-10 functions. Since IL-10 is a cytokine synthesis inhibitory factor, its absence could plausibly lead to enhanced inflammation or to immunopathologies, including autoimmune diseases.

In mice, the major cellular sources of IL-10 are $CD4^+$ Th2 and Treg cells, in addition to the monocyte/macrophage lineage, which includes some dendritic cell subsets. Unlike mice, all human $CD4^+$ subsets (Th0, Th1 and Th2 cells) can synthesize IL-10. IL-10, which can also be made by B cells, interferes with the biosynthesis of IL-12 (D'Andrea et al., 1993; Hsieh et al., 1993), a cytotoxic lymphocyte maturation factor and natural killer cell stimulatory factor. Blocking IL-12 production leads to inhibition of interferon-gamma (IFN-γ) production, a Th1 cytokine. IL-10 also inhibits the production of IL-2, a T cell growth factor (Taga and Tosato, 1992). In macrophages, IL-10 inhibits the production of the proinflammatory cytokines IL-1β, IL-6, and TNFα (Gerard et al., 1993; Minty et al., 1997; Ralph et al., 1992). The types of cytokines inhibited by IL-10 indicate why it is considered an anti-inflammatory cytokine and beneficial for pathologies induced by cell-mediated type-1 (Th1) responses and inflammation. An early report of IL-10 knockout mice demonstrated that absence of IL-10 causes inflammatory bowel disease (Kuhn et al., 1993). Following infection of IL-10 deficient mice by *Toxoplasma gondii*, overproduction of inflammatory cytokines resulted in death (Gazzinelli et al., 1996). In a model of spinal cord injury, IL-10 deficient mice displayed significantly more nerve damage early after injection of quisqualic acid (< 14 days) than did wild-type mice, and injection of IL-10 was found to be neuroprotective (Abraham et al., 2004).

In the mouse brain, IL-10 is not constitutively expressed, or else is expressed at very low levels. Analysis of gene expression using Affymetrix gene microarrays and real-time RT-PCR reveals that IL-10 is one of the lowest expressed cytokines in normal healthy mouse brains, whereas IL-18, IL-16, IL-15, and TGF- β are highly expressed (J. Kasten-Jolly and Lawrence, unpublished research). However, brain-specific IL-10 expression increases after CNS inflammation, in an attempt by the body to lessen the oxidative stress induced by the inflammatory cytokines. T cells from IL-10-deficient mice were unable to suppress active experimental autoimmune encephalomyelitis (EAE) which serves as a model for multiple sclerosis and is considered a Th1 cell-mediated autoimmune disease (Zhang et al., 2004). IL-10 is needed to suppress the IFNγ levels that enhance EAE neuropathology (Gonnella et al.,

2004). The source of the anti-inflammatory cytokines IL-10 and TGF- β, which attempt to regulate the neuroinflammation of EAE, appears to be Treg cells (Selvaraj and Geiger, 2008). IL-10 is known to induce suppressor of cytokine signaling protein (SOCS-3), a negative regulator of adaptive and innate immune responses that provides feedback attenuation of cytokine-induced immune and inflammatory responses in macrophages and microglia (Qin et al., 2007).

Since a substantial number of reports have demonstrated the involvement of IL-10 in immunoregulation through the use of exogenous IL-10 and neutralization of IL-10 activities with blocking antibodies, we can fairly safely conclude that the enhanced type-1 mediated immunopathological conditions observed in IL-10 knockout mice are due to the absence of IL-10. However, unless altered levels of IL-10, or modulation of its signaling, can be demonstrated via phenotype rescue or via confirmation with blocking antibodies or IL-1R depletion, the assumption cannot be made that IL-10 deficiency is responsible for altered neurobehaviors in IL-10 knockout mice.

5.2 Interleukin-10 knockout mouse behavioral studies

As detailed above, IL-10 knockout mice have been widely used to study the role of IL-10 in the peripheral and central immune responses; however, the behavioral profile of this knockout has not been well studied. In order to demonstrate the effectiveness of their newly developed QTL approach, Bolivar and colleagues (Bolivar et al., 2001a; Flaherty and Bolivar, 2007) screened a group of readily available knockout/congenic mouse strains for behavioral differences from B6. While some knockout/congenic strains were selected on the basis of previous reports that the mutant gene influences behavior, other strains, such as the interleukin-10 knockout/congenic (Kuhn et al., 1993), were selected based upon the absence of such behavioral connections. As an unexpected result of behavioral screening, a significant difference in open field behavior was established between the interleukin-10 knockout/congenic strain (B6-*Il10*$^{-/-}$; N10F14) and B6 (Stage #1). The low level of exploratory behavior observed in B6-*Il10*$^{-/-}$ mice was not found in (B6 × B6-*Il10*$^{-/-}$) F1 mice (Stage #2). The F1 mice did not behave differently in this task than B6 mice. This pattern is suggestive of a recessive behavioral trait, i.e., a single copy of the allele in question does not produce the phenotype.

The hypothesized contribution of the 129-derived flanking genes was assessed in a behavioral comparison of (129 × B6-*Il10*$^{-/-}$) F1 and (129 × B6) F1 mice (Stage #3B). Because the B6-*Il10*$^{-/-}$ behavioral phenotype is recessive, comparison of these two groups allows dissection of the relative contributions of the ablated and flanking genes, given the fact that the (129 × B6-*Il10*$^{-/-}$) F1 mice will have two copies of the flanking alleles and only a single copy of the mutant *Il10* gene. There was a difference between the two F1 groups, thus implicating 129-derived flanking genes, rather than *Il10* itself, in the behavioral deficit. Genotyping analyses of the genetic region encompassing *Il10* and its 129 flanking gene region on Chromosome 1 revealed that, in spite of extensive backcrossing, the knockout/congenic maintains a substantial segment of 129-derived genetic material (approximately 40 cM). Given the breeding scheme findings and considering that the region flanking the ablated *Il10* is polymorphic between B6 and 129, this study provides evidence supporting the existence of a QTL influencing open field behavior within a well-defined region on chromosome 1.

Once strong evidence has been obtained that the flanking region is responsible for the behavioral phenotype of interest, the next step is to identify the gene(s) responsible. In a recent paper, Rodrigues de Ledesma et al. (2006) sought to map the open-field activity QTL to a more refined set of 129 flanking genes in the B6-*Il10*$^{-/-}$ knockout/congenic strain. That goal was accomplished through behavioral assessment of B6-*Il10*$^{-/-}$ subcongenic strains of mice, which are generated by backcrossing to B6 and then selectively breeding recombinant mice that

exhibit recombination events within the flanking region. This method effectively breaks up the 129-derived flanking region, thereby permitting a more specific correlation between the open field activity phenotype and genotype at multiple genetic intervals within the flanking region. *Emo4*, an open field activity QTL, was mapped to a narrow region of chromosome 1 between 68.4 and 86.2 Mb. Surprisingly, the subcongenic-based analysis also resulted in the isolation of a second QTL, identified as *Reb1*. This novel QTL correlates with repetitive beam breaks in the open field and was mapped to a region of chromosome 1 between 91.4 and 100.0 Mb. With the candidate regions defined and narrowed, it is far easier to identify potential candidate genes. The candidates can then be assessed via compilation of previously reported expression and phenotype data, prioritizing by relevance to the observed phenotype, and then generation of a careful experimental approach to determine the gene(s) involved. The Rodrigues de Ledesma et al (2006) study is the first to illustrate the value of the approach developed by Bolivar and colleagues, indicating that a flanking gene effect, rather than the ablated gene, is responsible for a behavioral phenotype observed in a knockout/congenic strain. Importantly, this study shows that this classical mouse genetics approach to knockout/congenic phenotype analysis can be used to identify and map a QTL.

6. Conclusions

Knockout mouse technology radically transformed the biomedical sciences, changing the manner in which researchers approach the study of genes, proteins, and even biological systems. Given the central importance of this method, we must recognize its benefits but also its potential shortcomings. Due to the means by which many knockout mice are created, phenotypes originally attributed to the knocked out gene under study can actually be attributed to tightly-linked loci immediately flanking the ablated gene, or even to unlinked loci located throughout the genetic background of the knockout strain. In our research, we must always bear in mind these possible genetic confounds. This issue ultimately exists because the two strains used most often in the generation of knockout mice – B6 and 129 – are quite unlike each other genetically, physiologically, and behaviorally.

Various approaches have been suggested to address the issue of flanking and background genes, and some of the most widely used have been summarized here. B6 mice are typically used as experimental controls; therefore, the obvious solution to the issue is to render as much of the genetic background B6 as possible and to identify any polymorphic genetic regions. During any assessment of whether the ablation of a gene is, in fact, responsible for a phenotypic trait, the basic question should always be asked: is the observed phenotype relevant to what is known of the protein function? If the function of a gene is unknown, or if the observed phenotype deviates from what is reasonably anticipated, then the potential influence of 129-derived genes should be evaluated.

Acknowledgments

Support during the preparation of this article was provided by NIH grant MH068013. The authors also wish to thank Kevin Manley, Rose Auerbach and Lizbeth Varela Day (Wadsworth Center, Albany, NY) for helpful remarks and discussions during the preparation of the manuscript.

References

Abraham KE, McMillen D, Brewer KL. The effects of endogenous interleukin-10 on gray matter damage and the development of pain behaviors following excitotoxic spinal cord injury in the mouse. Neuroscience 2004;124:945–952. [PubMed: 15026134]

Bolivar VJ, Caldarone BJ, Reilly AA, Flaherty L. Habituation of activity in an open field: A survey of inbred strains and F1 hybrids. Behav Genet 2000;30:285–293. [PubMed: 11206083]

Bolivar VJ, Cook MN, Flaherty L. Mapping of quantitative trait loci with knockout/congenic strains. Genome Res 2001a;11:1549–1552. [PubMed: 11544198]

Bolivar VJ, Pooler O, Flaherty L. Inbred strain variation in contextual and cued fear conditioning behavior. Mamm Genome 2001b;12:651–656. [PubMed: 11471061]

Bothe GW, Bolivar VJ, Vedder MJ, Geistfeld JG. Genetic and behavioral differences among five inbred mouse strains commonly used in the production of transgenic and knockout mice. Genes Brain Behav 2004;3:149–157. [PubMed: 15140010]

Chandler KJ, Chandler RL, Broeckelmann EM, Hou Y, Southard-Smith EM, Mortlock DP. Relevance of BAC transgene copy number in mice: transgene copy number variation across multiple transgenic lines and correlations with transgene integrity and expression. Mamm Genome 2007;18:693–708. [PubMed: 17882484]

Collins FS, Rossant J, Wurst W. A mouse for all reasons. Cell 2007;128:9–13. [PubMed: 17218247]

Cook MN, Bolivar VJ, McFadyen MP, Flaherty L. Behavioral differences among 129 substrains: implications for knockout and transgenic mice. Behav Neurosci 2002;116:600–611. [PubMed: 12148927]

Crawley JN, Belknap JK, Collins A, Crabbe JC, Frankel W, Henderson N, Hitzemann RJ, Maxson SC, Miner LL, Silva AJ, Wehner JM, Wynshaw-Boris A, Paylor R. Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies. Psychopharmacology 1997;132:107–124. [PubMed: 9266608]

Crusio WE. Flanking gene and genetic background problems in genetically manipulated mice. Biol Psychiatry 2004;56:381–385. [PubMed: 15364034]

D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 1993;178:1041–1048. [PubMed: 8102388]

Ducottet C, Belzung C. Correlations between behaviours in the elevated plus-maze and sensitivity to unpredictable subchronic mild stress: evidence from inbred strains of mice. Behav Brain Res 2005;156:153–162. [PubMed: 15474660]

Flaherty, L.; Bolivar, V. Congenic and consomic strains. In: Jones, BC.; Mormede, P., editors. Neurobehavioral Genetics Methods and Applications. CRC Press; Boca Raton: 2007. p. 115-127.

Gazzinelli RT, Wysocka M, Hieny S, Scharton-Kersten T, Cheever A, Kuhn R, Muller W, Trinchieri G, Sher A. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. J Immunol 1996;157:798–805. [PubMed: 8752931]

Gerard C, Bruyns C, Marchant A, Abramowicz D, Vandenabeele P, Delvaux A, Fiers W, Goldman M, Velu T. Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. J Exp Med 1993;177:547–550. [PubMed: 8426124]

Gerlai R. Gene-targeting studies of mammalian behavior: is it the mutation or the background genotype? Trends Neurosci 1996;19:177–181. [PubMed: 8723200]

Gonnella PA, Waldner HP, Kodali D, Weiner HL. Induction of low dose oral tolerance in IL-10 deficient mice with experimental autoimmune encephalomyelitis. J Autoimmun 2004;23:193–200. [PubMed: 15501390]

Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science 1993;260:547–549. [PubMed: 8097338]

Kalueff AV, Tuohimaa P. Contrasting grooming phenotypes in C57Bl/6 and 129S1/SvImJ mice. Brain Res 2004;1028:75–82. [PubMed: 15518644]

Kibar Z, Gauthier S, Lee SH, Vidal S, Gros P. Rescue of the neural tube defect of loop-tail mice by a BAC clone containing the *Ltap* gene. Genomics 2003;82:397–400. [PubMed: 12906864]

Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell 1993;75:263–274. [PubMed: 8402911]

McFadyen MP, Kusek G, Bolivar VJ, Flaherty L. Differences among eight inbred strains of mice in motor ability and motor learning on a rotorod. Genes Brain Behav 2003;2:214–219. [PubMed: 12953787]

Minty A, Ferrara P, Caput D. Interleukin-13 effects on activated monocytes lead to novel cytokine secretion profiles intermediate between those induced by interleukin-10 and by interferon-gamma. Eur Cytokine Netw 1997;8:189–201. [PubMed: 9262968]

Montkowski A, Poettig M, Mederer A, Holsboer F. Behavioural performance in three substrains of mouse strain 129. Brain Res 1997;762:12–18. [PubMed: 9262153]

Nagy, A.; Gertsenstein, M.; Vintersten, K.; Behringer, R. Maniulating the Mouse Embryo. Cold Spring Harbor Laboratory Press; Cold Spring Harbor: 2003.

Papaioannou, VE.; Behringer, R. Mouse Phenotypes: A Handbook of Mutation Analysis. Cold Spring Harbor Press; Cold Spring Harbor: 2004.

Paylor R, Crawley JN. Inbred strain differences in prepulse inhibition of the mouse startle response. Psychopharmacology 1997;132:169–180. [PubMed: 9266614]

Qin H, Roberts KL, Niyongere SA, Cong Y, Elson CO, Benveniste EN. Molecular mechanism of lipopolysaccharide-induced SOCS-3 gene expression in macrophages and microglia. J Immunol 2007;179:5966–5976. [PubMed: 17947670]

Ralph P, Nakoinz I, Sampson-Johannes A, Fong S, Lowe D, Min HY, Lin L. IL-10, T lymphocyte inhibitor of human blood cell production of IL-1 and tumor necrosis factor. J Immunol 1992;148:808–814. [PubMed: 1730874]

Ridgway WM, Healy B, Smink LJ, Rainbow D, Wicker LS. New tools for defining the 'genetic background' of inbred mouse strains. Nat Immunol 2007;8:669–673. [PubMed: 17579641]

Rodrigues de Ledesma AM, Desai AN, Bolivar VJ, Symula DJ, Flaherty L. Two new behavioral QTLs, Emo4 and Reb1, map to mouse Chromosome 1: Congenic strains and candidate gene identification studies. Mamm Genome 2006;17:111–118. [PubMed: 16465591]

Sechler JM, Yip JC, Rosenberg AS. Genetic variation among 129 substrains: practical consequences. J Immunol 1997;159:5766–5768. [PubMed: 9550369]

Selvaraj RK, Geiger TL. Mitigation of experimental allergic encephalomyelitis by TGF-beta induced Foxp3+ regulatory T lymphocytes through the induction of anergy and infectious tolerance. J Immunol 2008;180:2830–2838. [PubMed: 18292504]

Seong E, Saunders TL, Stewart CL, Burmeister M. To knockout in 129 or in C57BL/6: that is the question. Trends Genet 2004;20:59–62. [PubMed: 14746984]

Silver, L. Mouse Genetics: Concepts and Applications. Oxford University Press; New York: 1995.

Simpson EM, Linder CC, Sargent EE, Davisson MT, Mobraaten LE, Sharp JJ. Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. Nat Genet 1997;16:19–27. [PubMed: 9140391]

Taga K, Tosato G. IL-10 inhibits human T cell proliferation and IL-2 production. J Immunol 1992;148:1143–1148. [PubMed: 1737931]

Threadgill DW, Yee D, Matin A, Nadeau JH, Magnuson T. Genealogy of the 129 inbred strains: 129/SvJ is a contaminated inbred strain. Mamm Genome 1997;8:390–393. [PubMed: 9166580]

van Bogaert MJ, Groenink L, Oosting RS, Westphal KG, van der Gugten J, Olivier B. Mouse strain differences in autonomic responses to stress. Genes Brain Behav 2006;5:139–149. [PubMed: 16507005]

Wahlsten D, Ozaki HS, Livy D. Deficient corpus callosum in hybrids between ddN and three other abnormal mouse strains. Neurosci Lett 1992;136:99–101. [PubMed: 1635672]

Wolfer DP, Crusio WE, Lipp HP. Knockout mice: simple solutions to the problems of genetic background and flanking genes. Trends Neurosci 2002;25:336–340. [PubMed: 12079755]

Zhang X, Koldzic DN, Izikson L, Reddy J, Nazareno RF, Sakaguchi S, Kuchroo VK, Weiner HL. IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25+CD4+ regulatory T cells. Int Immunol 2004;16:249–256. [PubMed: 14734610]

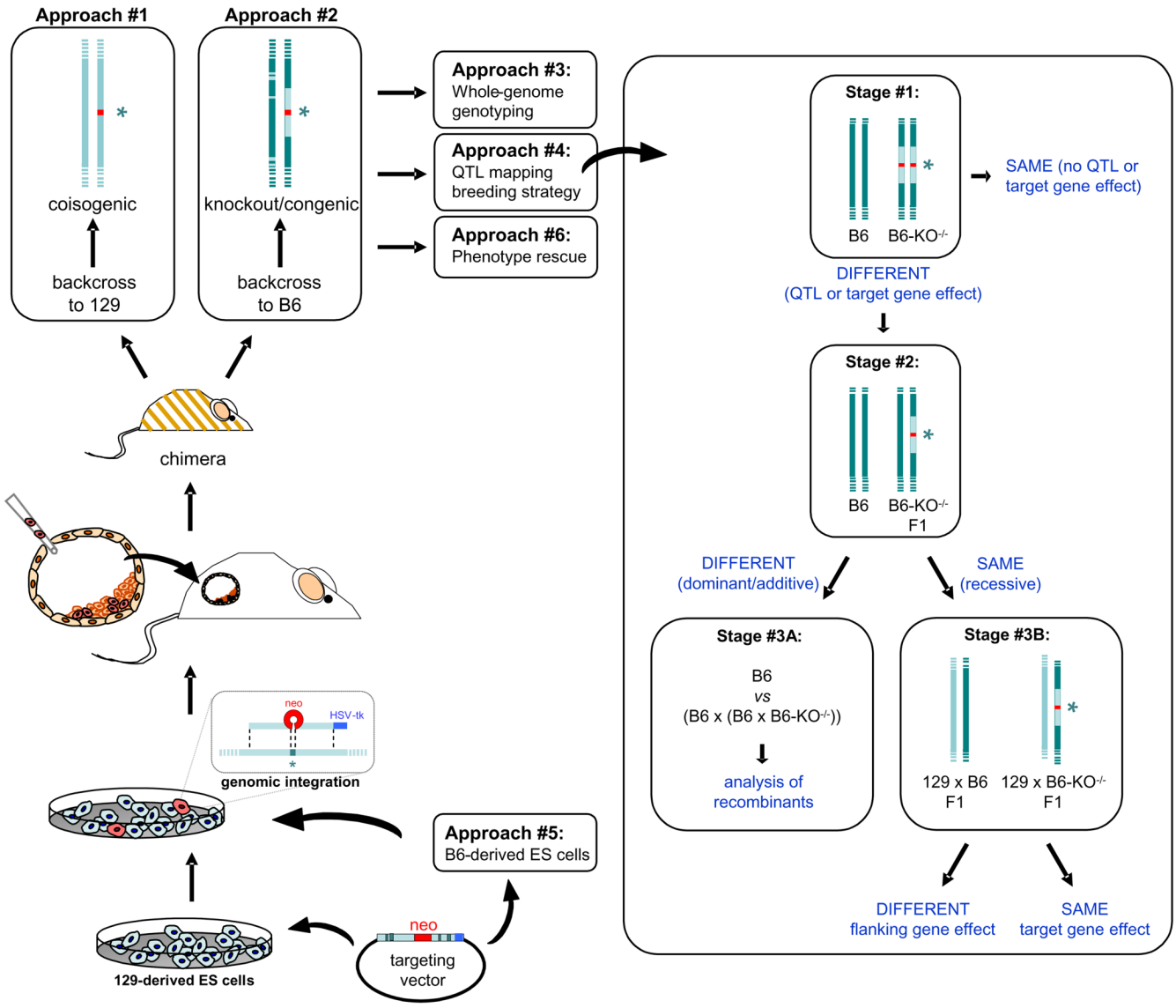

Figure 1.

Schematic of approaches that address potential genetic confounds associated with knockout gene characterization. The basic method for developing a knockout mouse is illustrated in the left hand side of the figure. 129-derived ES cells are typically used in the generation of gene-targeted mice. The targeting vector containing the mutant gene of interest is electroporated into the ES cells, where the mutant gene integrates into the genome by homologous recombination. ES cells carrying the mutation are then injected into blastocysts, which are in turn injected into pseudopregnant females. The resulting chimeric mice undergo selective breeding. As there are potential problems with determining whether an observed phenotype is due to the ablated gene or other 129 genes (see text for details) a number of approaches to combat this problem have been developed. Six approaches are represented here. First, the null mutation can be maintained on the same 129 genetic background as the ES cells, thus producing a coisogenic strain (Approach #1). Second, a knockout/congenic strain can be generated by backcrossing the knockout mouse to B6 for more than 10 generations (Approach #2). This process maintains the null mutation on a primarily B6 background, but multiple regions of 129 ES cell-derived genetic material remain. The role of the ablated gene in the observed phenotype(s) may be

clarified by employing SNP-based genotyping throughout the genome (Approach #3), quantitative trait loci mapping (Approach #4), or phenotypic rescue (Approach #6). Finally, the use of B6 rather than 129 ES cells completely eliminates the 129-background gene issue (Approach #5).