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Mutagenesis as an unbiased approach to identify novel contraceptive targets

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Abstract

To accommodate diverse personal needs in family planning, diverse contraceptive approaches are desirable. This goal requires identification of new contraceptive targets. Phenotype-driven mutagenesis is an unbiased approach to identify novel genes and functions in reproductive processes. The ReproGenomics Program at The Jackson Laboratory is a United States National Institutes of Health resource for production, identification and distribution of mutant mouse models of infertility that can be used for identification of potential targets for contraception. The strategy of this program is whole genome, random ENU mutagenesis, coupled with a phenotype screen for breeding failure as the only phenotype. A three-generation breeding scheme selects recessive mutations affecting reproductive functions. G3 males and females that fail to reproduce by natural mating to wild-type animals undergo secondary phenotype screens to assess gonad and accessory organ histology, hormone production, gamete production and gamete function in fertilization. The genetic transmission of the infertility trait in each family is confirmed and each mutation is genetically mapped to a defined chromosome region, facilitating identification of candidate genes from sequence and expression databases. Genes essential for fertility in both males and females and acting both meiotically and post-meiotically have been identified by this strategy. Phenotypes include male infertility with normal sperm count, but failure in fertilization of oocytes. Phenotype descriptions of each mutation are posted on the program website, http://reprogenomics.jax.org. These unique reproductive mutant mouse resources will lead to new discoveries in andrology (and gynecology) research, as well as reproductive medicine. Dissection of gene function in known and newly discovered reproductive pathways will expand our focus to reveal novel targets for contraception.

Keywords: Mutagenesis; Contraception; Infertility; Gametogenesis; Fertilization; Mouse models

1. Introduction

Until now, development of contraceptives has been founded on experimentally based knowledge of the roles of specific molecules in reproduction. This has led to considerable success for contraceptive interference with hormone-mediated pathways leading to gamete production and/or reproductive behavior and many efforts at immunocontraception (Nass and Strauss, 2004). These similar approaches are biased by what we already know or infer about the genes and proteins that play roles in reproductive function. However, sequencing of mammalian genomes has revealed that a significant proportion of the putative coding sequences represent unknown genes, suggesting that there could be far more genes that play roles in reproduction than we have suspected. Novel approaches to male contraception require identification of "novel" (meaning previously unsuspected) proteins critical for male reproduction. For this reason, intense effort is currently devoted to identification of transcriptomes and proteomes of male germ cells and accessory reproductive organs (Chauvin and Griswold, 2004; Schultz et al., 2003; Shima et al., 2004). These studies are yielding a tremendous amount of new information, some quite surprising, about the multitude of genes and proteins expressed in male reproductive tissues. For example, microarray analyses suggest that 4% of the mouse genome is expressed specifically and uniquely in male germ cells, and that most of these genes are expressed post-meiotically (Schultz et al., 2003). The "-omics" of male reproductive function will soon be extended into defining the reproductive glycome and lipidome (Nass and Strauss, 2004). There is no doubt that these molecule profiling and "harvesting" strategies are useful, but the primary caveat is that these approaches provide no information about the requirement for any of the identified gene products in sperm function and fertility.

At The Jackson Laboratory, the ReproGenomics Program is founded on a "phenotype-based" rather than "expression-based" strategy for discovery of genes involved in fertility, which may

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then identify novel contraceptive targets (Lessard et al., 2004). Briefly, we conduct random, whole-genome mutagenesis, produce mice that are homozygous for a given recessive mutation, and then ask the mouse to tell us if the mutated gene is required for reproductive function, by screening for its fertility in natural mating. This phenotype-driven approach harvests fewer genes than do transcriptome or proteome profiling studies, but, importantly, we know at the outset that each gene identified by this strategy is essential for male (or female) reproductive function.

2. Overview of ReproGenomics: hierarchical phenotype screening and gene identification

N-Ethyl-*N*-nitrosourea (ENU), a potent germ cell mutagen in mice (Noveroske et al., 2000), is used to create random, genome-wide germ cell base-pair mutations in male C57BL6/J (B6) mice. To facilitate eventual mapping of induced mutations with DNA polymorphisms, these males are crossed to C3HeB/FeJ (C3H) females to produce the G1 generation. Each G1 male found a family segregating a unique spectrum of mutations that were present in the fertilizing spermatozoon from the mutagenized B6 male. The G1 males are crossed to C3H females and G2 daughters crossed to the G1 male in order to produce the G3 generation. One-fourth of the individuals in the G3 generation will be homozygous for any recessive mutation inherited from the G1 male. This mating scheme is illustrated in Fig. 1.

The first tier of the hierarchical phenotype screen is to mate all G3 individuals to wild-type mice. In this manner, we determine if any of the induced mutations produce an infertility phenotype, as any individual (male or female) that fails to reproduce is a candidate for carrying a homozygous recessive mutation affecting reproduction. In the second tier of the male phenotype screen (Fig. 2), the cause of infertility is defined more precisely by analyses of gamete function during in vitro fertilization (IVF) and histological examination of reproductive tissues; a similar screen is used to define female infertility phenotypes. This information facilitates discrimination among mutations with phenotypes

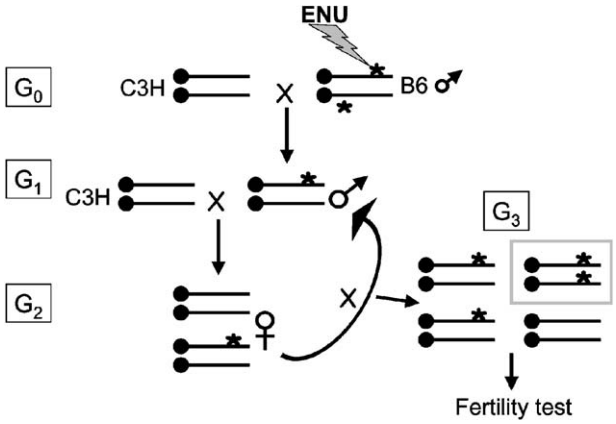

Fig. 1. This diagram presents the scheme for ENU mutagenesis and breeding to produce G3 individuals, all of which are tested for fertility in natural mating.

affecting reproductive behavior, gametogenesis, post-gonadal gamete maturation, or gamete function in fertilization processes.

The essential final step in this endeavor is the fine mapping and positional cloning of each mutated gene of interest. This process entails classical genetic mapping by recombination analysis, followed by testing of candidate genes identified from sequence analysis. Detection of a mutation in the sequence of a candidate gene is a strong indication that the gene has been identified and this can be confirmed by expression analyses, transgenic rescue and other genetic and molecular approaches to ablate or knockdown the function of the normal gene. The positional cloning procedure sometimes can be arduous, but is essential in identifying a promising contraceptive target.

In little over 2 years, this program has already generated more than 30 mutant mouse models of infertility, with phenotypes observed in males only, females only, or both sexes. Although not initially expected, the program has identified many more mutations that affect males only than ones that affect females only; other small-scale mutagenesis screens for fertility mutations have reported the same phenomenon

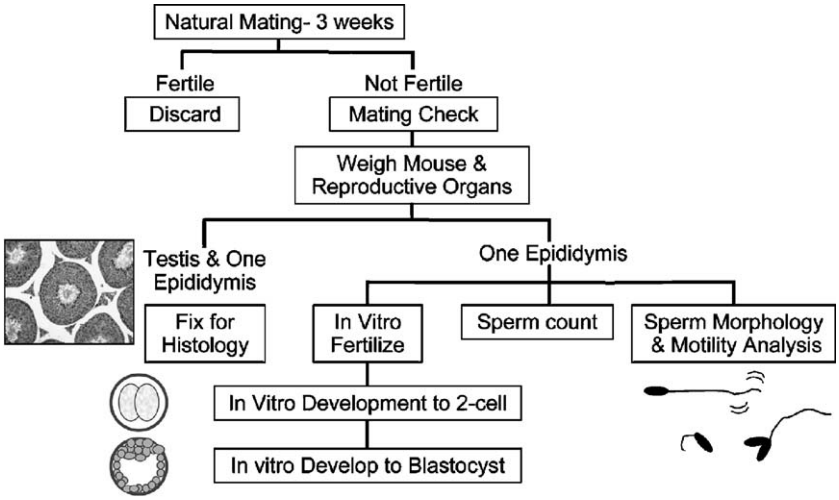

Fig. 2. This figure presents a flow chart of the diagnostic work (secondary screen) performed on male individuals found to be infertile by the primary screen. These analyses help pinpoint the cause for infertility and aid in identifying phenotypes that may be useful for contraceptive development.

Fig. 3. This screen view of the homepage for the ReproGenomics website (http://reprogenomics.jax.org/) illustrates that interested individuals can enter and navigate from several points, including phenotypes, genomic map position and mutant list. Instructions for obtaining mutant mouse models are provided.

(Clark et al., 2004). The ReproGenomics program website (http://reprogenomics.jax.org/) presents the entire list of mutations and candidate genomic map positions, updated regularly and searchable from several different entry points, including phenotype and regional position on the genomic map (Fig. 3). Carrier breeding pairs for any of the posted mutations are available free of charge to any interested investigators.

3. ReproGenomics: mutations in "old" and "new" genes

To assess efficacy and efficiency of this program, two questions can be asked: have mutations been detected in genes known to cause infertility? and have mutations been detected in novel genes? These questions address two important aspects of validation of the approach. First, we want to know that we are getting full genome coverage with mutations and that the single-base-pair mutagenesis strategy can produce phenotypes from mutations in genes previously known to affect fertility. Second, we want to know that the strategy can also target previously unknown genes, as this is a route to novel contraceptive approaches. The answer to both questions is, satisfyingly, yes: we have induced mutations in genes previously known to affect fertility, and we have identified seemingly novel genes by this approach. These findings are described in more detail below.

The *repro11* mutation is an example of a new mutation in an "old" gene. Its mutant phenotype is male and female infertility. Mutant males have small testes due to arrest of spermatogenesis at the primary spermatocyte stage. Preliminary mapping placed the *repro11* mutation in a region of Chr 15 that included the *Smc1b* (also known as *Smc1l2*) gene encoding a meiosis-specific cohesin protein, responsible for holding sister chromatids together. This gene had previously been knocked-out by a targeted approach; the knockout phenotype is similar if not identical to the phenotype of the new *repro11* mutation (Revenkova et al., 2004). Thus, *Smc1b* was an excellent candidate for the gene mutated by the *repro11* mutation, and it was sequenced in the DNA of homozygous affected males. This effort revealed that the *repro11* mutation is in the *Smc1b* gene, demonstrating that the mutagenesis strategy does indeed identify genes previously known to be involved in fertility. The existence of a new, point mutation in the *Smc1b* gene will facilitate future protein structure–function analyses to more precisely determine the role of this interesting chromosomal cohesin in germ cells.

More importantly, "new" genes are being detected by the ReproGenomics mutations. In fact, in contrast to the situation for *repro11*, the majority of mutations produced have been mapped to chromosome regions that do not harbor genes previously known to be involved in fertility. In these cases, once a mutation is mapped to a region of approximately 10 Mb, database analysis is used to examine each of the included genes (perhaps 100 or more). In this manner, we determine if any are known to be involved in reproduction. In most cases, none have a demonstrated role in fertility; however, analysis of expression databases frequently reveals gonad expression of one or more genes in the candidate genomic interval. These analyses thus demonstrate the efficacy of the mutagenesis and phenotype screening strategies

for producing mutations in genes that are absolutely required for fertility (the basis of the phenotype screen), but not previously known to be required for reproduction.

4. ReproGenomics: male infertility phenotypes and progress toward contraceptive targets

Male infertility phenotypes identified by ReproGenomics are diverse and include both gonadal and post-gonadal defects (http://reprogenomics.jax.org/). Mutations that affect spermatogenesis include phenotypes of germ cell or spermatogonial depletion, impairment of meiotic functions (Bannister et al., 2004), or disruption of spermiogenic processes, manifesting as defective spermatid morphology. Collectively, phenotypes of these mutations are azoospermia or oligoasthenozoospermia. Thus, they are excellent models for the study of human male infertility syndromes, and will likely yield important new information about sperm morphogenesis. Nonetheless, these mutations, by virtue of their extreme phenotypes, may not identify appropriate or desirable contraceptive targets.

Infertility phenotypes of more interest for identification of sperm contraceptive targets are those that do not affect sperm numbers or morphology, but produce post-gonadal defects in sperm function. These phenotypes could be defective sperm motility, defective sperm function in fertilization (as assessed by IVF), or unexplained male infertility. An example of one such mutation identified by the ReproGenomics program is fertilization failure 1 (*ferf1*). Affected *ferf1*/*ferf1* males are normal with respect to many physiologically important (and desirable) parameters: body weight, sexual behavior, sperm count and morphology. The sole apparent phenotype of homozygous *ferf1* male mice is failure to produce offspring when bred to normal females. The mutant males produce a copulatory plug; therefore, sexual behavior appears normal. When used in IVF, sperm from affected males fail to fertilize zona pellucida-enclosed oocytes. However, mutant sperm fertilize and activate zona-free oocytes at rates comparable to that of normal, wild-type sperm. Thus, sperm in *ferf1*/*ferf1* males appear to be impaired in a process leading to fertilization. This could be an event in epididymal maturation, a process involved in the acrosome reaction, or a process involved in sperm-egg recognition and binding. Thus, knowledge of the protein affected by the *ferf1* mutation could yield insight into poorly understood steps in capacitation and/or fertilization and possibly identify a novel contraceptive target. The *ferf1* mutation has been fine mapped to a small region on Chr 14 encompassing 34 genes, none of which have been known to be required for fertility. Among these genes, several are expressed in testes and have human homologs, making them interesting candidates for harboring the *ferf1* mutation. The challenging opportunity now is final identification of the gene interrupted by the *ferf1* mutation and subsequent experimental analyses to link the protein product to processes involved in sperm function.

5. Conclusions and outlook

ReproGenomics is founded on the premise that novel contraceptive targets can be identified through discovery of mutations with the singular phenotype of infertility. The ultimate success of mutagenesis strategies for identification of new targets for contraception will depend on unbiased phenotype screens, as described here. The program has already identified mutant male mice that have no apparent adverse phenotypes or gonadal abnormalities, but whose sperm do not function in fertilization. We expect that more such mutant models will be produced by the program and that, collectively, they will provide entrée points for defining processes amenable to contraceptive interference. Indeed, one good contraceptive could justify the entire program, but, in fact, the program's value extends further and deeper in providing models for understanding human reproductive health, both male and female.

How do we get from the mutation to the contraceptive? Clearly, identification of a promising mutant phenotype and the gene product are only the first steps in a process of contraceptive development. At the simplest level, a protein involved in fertilization (or other reproductive) functions can be targeted by immunocontraception. Recent history, however, suggests that this approach can be fraught with complications and/or not as effective as originally anticipated; thus pharmaceutical companies are not likely to be interested in this strategy. A more likely approach would be exploitation of libraries of small molecule inhibitors to target any step in the pathway from synthesis to function of a protein identified as playing a role in male reproduction. In fact, mutagenesis may define domains of a protein (which could be ubiquitous) that have a fertility-specific function. However, the ultimate identification of a useful contraceptive target from infertility mutations will emerge from comprehensive experimental analyses of the biology and chemistry of the affected protein identified by the male infertility phenotype. When and where is it expressed? Testis or epididymis or other tissue? In germ cells or supporting somatic cells? Where within expressing cells does the protein localize? What signals are required to get it to its site of action? Is the protein post-translationally modified, and if so, by what pathway? What sites on the protein are required for its activity? What are the interacting partners of the critical protein? How are these interactions established? These are but a few of the critical questions. Approaches founded on the identification of a critical protein, but encompassing these holistic perspectives, will reveal avenues that can be exploited in contraceptive development.

Contraception should accommodate diverse personal, cultural and family needs. For this reason, a desirable societal endpoint is availability of contraceptive choices. To reach this endpoint will require imaginative and unbiased strategies to identify a multitude of contraceptive targets, the tenacity to explore many of these and the will to invest in contraceptive development. Mutagenesis, as demonstrated by the ReproGenomics program, is an intellectually sound and strong first step toward these important goals.

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