TECHVIEW: MOLECULAR GENETICS

# MaRX: An Approach to Genetics in Mammalian Cells

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A genetic approach is the most direct way to elucidate biological processes that are poorly understood. One of the first such efforts—the landmark study of Beadle and Tatum (*1*) on the genetics of metabolic pathways—established the influential "one gene, one enzyme" hypothesis. In subsequent decades, the yeasts *Saccharomyces cerevisiae* and *S. pombe* became the premier genetic models. The oft-touted "power of yeast genetics" was not fully realized, however, until classical techniques were combined with an ability to manipulate the organisms with recombinant DNA methods (*2–4*). Thus were conceived the tools that today make yeasts the best-characterized eukaryotes. These tools, however, have limitations: accumulating human sequence data reveals many genes that are not represented in yeast. How can the leap be made from yeast to human?

To solve this problem, we sought to apply genetic methods to mammals or their manipulable surrogates, cultured mammalian cells. Rather than creating a genetic methodology that technically mirrors the approach in yeast, we developed one with comparable genetic access to mammalian biology—the MaRX system.

To date, the application of molecular genetics to cultured mammalian cells—probably the most widely used model "organisms" in mammalian biology—has proved problematic. Only sporadic attempts have been made (*5–10*), despite the fact that many disease-related processes can be readily investigated in tissue culture. Because they are asexual diploids, mammalian cells are inaccessible to all classical genetic methods except mutagenesis.

A variety of technical barriers impede the use of genetic cloning approaches in mammalian cells. First, the inability to perform genetic crosses prevents the creation of complex mutant cell strains. Second, mammalian cells in culture are more plastic than are free-living microorganisms. Thus, significant variation occurs even among cells derived from a clonal isolate. Ultimately, this translates into phenotypes that are more prone to spontaneous reversion than those used for genetic selection in microorganisms. Finally, the vectors currently available for genetic manipulation of cultured cells are relatively primitive.

We therefore developed the MaRX system, a specialized strategy to facilitate function-based gene isolation in mammalian cells. This system relies on two concepts to overcome impediments to the use of genetic methods in cultured cells. First is the use of nucleic acid as a "virtual mutagen" rather than reliance on chemical or other mutagens. Cloned complementary DNAs (cDNAs), either in the sense or antisense orientation, are used to reversibly alter gene expression thereby creating a phenotype in a cultured cell. Second, the tendency of such phenotypes to revert spontaneously has been accommodated. The system allows efficient introduction of cDNA libraries into target cells, and allows efficient recovery of either individual genes or complex sublibraries from cell populations that have been enriched on the basis of a specific biological characteristic. As shown in Fig. 1, the essence of the approach is the ability to rapidly filter complex mixtures of clones through multiple rounds of phenotypic selection, termed cycle cloning.

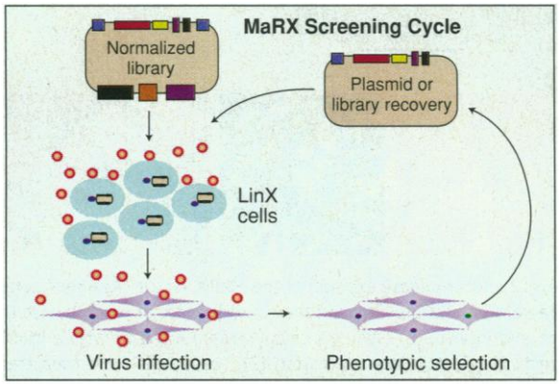


**Fig. 1.** The MaRX cycle. A normalized DNA library is converted into a library of retroviruses by using a packaging cell line (linX). Then, these infected recipient cells are selected or enriched on the basis of a specific biological property. Proviruses are recovered and used for virus production and subsequent rounds of screening.

The difficulty of manipulating large numbers of tissue culture cells coupled with the need to screen complex libraries dictates the need for efficient gene transfer. To achieve this goal in a wide range of cell types, we relied on newly designed, replication-deficient retrovirus vectors. The genomic structure and replication of these viruses is well understood, thus simplifying modification of existing systems for use as genetic tools. Furthermore, stable integration of recombinant retroviruses allows phenotypes to be assessed over many cell generations. However, downstream analysis of cDNAs that elicit selected phenotypes is complicated by the need to recover a single-copy provirus from the host genome. Previous applications of the retroviral vectors for functional cloning have relied on polymerase chain reaction (PCR) for isolation of virus-borne cDNA fragments (*6*, *7*, *10*). This approach may be sufficient when only a few cell clones need to be analyzed. However, PCR-based approaches are ill suited for manipulation of complex populations.

We addressed the problem of efficiently recovering integrated retroviruses by incorporating into MaRX the ability to be excised from genomic DNA by the action of a site-specific recombinase either in vitro or in vivo (Fig. 2A). The effect of excision in vivo is loss of the integrated virus. This provides a simple mechanism to demonstrate that the phenotype of a selected cell requires expression of the exogenous genetic element—a reversion test (Fig. 2B). Excision in vitro is accomplished by treating purified genomic DNA with the appropriate recombinase. This generates a circular molecule carrying the sequence responsible for generating the desired phenotype. To facilitate recovery of this excised virus, we have included within the MaRX provirus an optimized mini-plasmid (~700 base pairs in length). Thus, the excised provirus can be rescued simply by transforming recombinase-treated genomic DNA into highly competent *Escherichia coli*. This excision protocol also allows recovery of individual genes or complex mixtures of proviruses.

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The excised MaRX provirus has a single intact long-term repeat sequence. Upon transfection into an appropriate packaging cell line, the recovered provirus yields infectious retrovirus with an efficiency similar to that of the intact MaRX vector. Therefore, verification of a genetic rescue can be achieved without manipulation of individual candidate fragments. The ability to rescue a library of functional proviruses from a selected cell population allows a gene to be enriched from complex mixture through multiple rounds of phenotypic selection (through use of a single cell line; multiple, different cell lines; or selection criteria). Because many interesting phenotypes are "leaky," the ability to pass complex populations through multiple rounds of selection (cycle cloning) allows access to a wider range of biological problems.

The primary motivation for creating the MaRX system was to enable the cloning of mammalian genes by relying solely on their functional properties. To test the efficacy of our approach, we sought to reproduce one of the first marker rescue experiments to succeed in cultured cells, the cloning of the *ras* oncogene (*8*).

NIH-3T3 cells were infected with a MaRX cDNA library derived from a tumor cell line. A screen for transformation yielded a number of foci. Many of the isolates that displayed the most highly transformed phenotype carried MaRX proviruses encoding activated *ras* alleles. In a screen that took only 8 weeks, *ras* was isolated four independent times from 20 standard (100 mm) tissue culture plates. Thus, this simple model verified our ability to isolate relevant genes through phenotypic selection.

The ability to probe the function of specific genes through the creation of loss-of-function "alleles" is at the heart of any genetic methodology. The diploid nature of mammalian cells necessitates the use of unconventional approaches to the creation of "recessive" mutants. Effective inhibition of gene function can occur following expression of antisense RNAs (*11*). We used this method to test the MaRX system's ability to assess the consequences of loss of the tumor suppressor gene *p53*.

We created a directional, randomly primed cDNA library consisting of fragments of the *p53* coding sequence, because antisense mRNA fragments may inhibit gene expression more effectively than complete antisense mRNAs (*10*). This library was transferred into A3 cells, a murine embryonic fibroblast (MEF) derivative that ectopically expresses a conditional version of *p53* from a strong viral promoter. Shift of these cells to the permissive temperature resulted in cell-cycle arrest. Infection of A3 cells with the antisense *p53* mini-library allowed colony formation at a frequency of roughly $10^{-3}$ per cell.

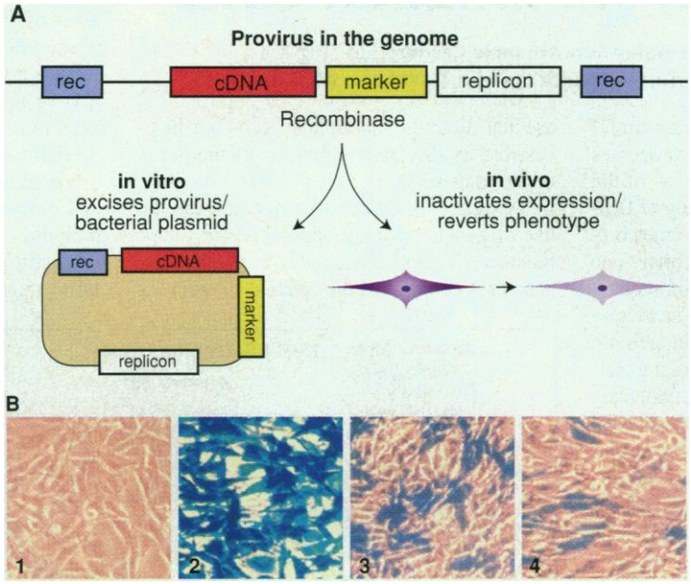


**Fig. 2.** Recombinase excision of the MaRX vector. **(A)** A representation of the integrated MaRX provirus and the consequences of recombinase treatment in vitro and in vivo. **(B)** NIH-3T3 cells (1) were infected with a MaRX virus that directs β-galactosidase expression (2). These cells were then transduced with a retrovirus that directs recombinase expression (3). Upon recombinase expression and continued passage (4), the phenotype of these cells (β-galactosidase expression) is reverted.

Proviruses containing interfering *p53* fragments were recovered from clones that resisted growth arrest. Expression of the most highly represented fragments resulted in a >90% inhibition of p53 protein expression and in efficient rescue of growth arrest. In primary cells (MEF cells), the selected fragments inhibited expression of endogenous p53, extended life-span, and protected the cell from DNA-damaging agents. Excision of the p53-inhibitory provirus (the "virtual mutagen") from any of these cell populations reverted the p53-null phenotype, demonstrating a continuous dependence on the antisense RNA. Expression of a full-length *p53* antisense RNA produced effects that were indistinguishable from those seen with the selected fragments. Thus, at least in this instance, use of a restricted antisense gene fragment was not required to generate a phenotype.

These results demonstrate that the MaRX system can create reversible, loss-of-function phenotypes. Antisense RNAs can work effectively against endogenous mRNAs and can even inhibit expression from ectopically expressed transcripts.

We have further validated the MaRX system in a range of biological contexts. For example, we investigated the roles of tumor suppressor function in cellular senescence, studying multiple genes and their interactions, and of inhibitory cytokines in growth control (*12, 13*). A search for genes that protect from oncogene-dependent cell death revealed a potential oncogene (*14*), and a screen for bypass of *p53* function uncovered a possible explanation for the long-mysterious link between chronic inflammation and cancer (*15*). We even deployed the MaRX system to identify genes that confer resistance to widely used pharmaceuticals (*16*). The versatility of the approach is exemplified by adaptation of the MaRX system to create two different versions of a high-throughput, homologous, mammalian secretion trapping system that is capable of identifying type I, type II, and unconventional secretion signals (*17*).

We have created a coherent system of reagents that enables a powerful genetic screening approach to a broad range of biological problems in mammalian cells. In principle, this system can be used to investigate any aspect of biology or pathobiology that can be recapitulated in a cell-culture model. In due course, we anticipate elaboration of MaRX into a recombinant mutagen for whole-animal studies.

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