Manipulating the mammalian genome by homologous recombination

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Gene targeting in mammalian cells has proven invaluable in biotechnology, in studies of gene structure and function, and in understanding chromosome dynamics. It also offers a potential tool for gene-therapeutic applications. Two limitations constrain the current technology: the low rate of homologous recombination in mammalian cells and the high rate of random (nontargeted) integration of the vector DNA. Here we consider possible ways to overcome these limitations within the framework of our present understanding of recombination mechanisms and machinery. Several studies suggest that transient alteration of the levels of recombination proteins, by overexpression or interference with expression, may be able to increase homologous recombination or decrease random integration, and we present a list of candidate genes. We consider potentially beneficial modifications to the vector DNA and discuss the effects of methods of DNA delivery on targeting efficiency. Finally, we present work showing that gene-specific DNA damage can stimulate local homologous recombination, and we discuss recent results with two general methodologies—chimeric nucleases and triplex-forming oligonucleotides—for stimulating recombination in cells.

Homologous recombination (HR) provides a precise mechanism for targeting defined modifications to genomes in living cells. In the 15 years since gene targeting was demonstrated in vertebrate cells (1–4), it has been used extensively to investigate gene function and to create mouse models of human diseases. Thus, gene targeting is now a standard tool of somatic cell genetics, as it has been in yeast for many years. Calling it a standard tool, however, does not mean that gene targeting is easy or that success is assured. Indeed, its application requires a certain persistence of effort that is not necessary, for example, in *Saccharomyces cerevisiae*. Any approach that would simplify the process in mammalian cells would be welcomed. Does our current knowledge of recombination in somatic cells offer any promising new strategies for gene targeting? We address this question here.

Various aspects of HR and nonhomologous end joining (NHEJ) have been covered in recent reviews (5–10), as have strategies for gene targeting (11–16). Space limitations preclude discussion of other promising approaches to gene correction, including targeting with small DNA fragments (17, 18) and RNA/DNA chimeras (19, 20).

Current protocols for gene targeting rely on the cell's enzymatic machinery to accomplish HR, which generally occurs at a frequency of roughly one event per 10^5 to 10^7 treated cells (14). This low frequency of targeting probably reflects an average low frequency of recombination in every cell, rather than the presence of rare, HR-competent cells in the population. Early experiments using microinjection obtained targeted recombinants at about 1 per 1,000 injected cells (2). Moreover, recent experiments designed to stimulate HR, as discussed below, generated recombinants in several percent of treated cells (21). An average capability per cell is, of course, an oversimplification because there are clear indications of cell cycle-dependent and damage-induced expression of proteins involved in recombinational processes (5, 22–25)

The principal barrier to facile gene targeting in vertebrate cells is not the low frequency of HR, but rather the high frequency of random (nonhomologous) integration, which occurs in about one cell per 10^2 to 10^4 treated cells (26). For most cells, targeted recombinants are obscured by more than a 1,000-fold higher frequency of random integrants (14). Random integration is thought to occur by NHEJ, although analysis of multiple integration junctions indicates that more homology is used than is common for NHEJ (27). Several tricks have been devised to suppress the number of random integrants that survive selection and thereby improve the ratio of targeted recombinants to random integrants. These include positive-negative selection, promoter and polyadenylation trap strategies, and marker-target gene fusions (28–30). Positive-negative selection—the most commonly used approach—works well in mouse embryonic stem (ES) cells and has made gene targeting fairly routine in those cells.

For many purposes, it would be useful to target genes in established cell lines, which are widely used as model systems. With rare exceptions (31) positive-negative selection in cell lines enriches targeted recombinants less than 5-fold relative to random integrants (32). This low degree of enrichment, coupled with the lower starting ratio of targeted recombinants to random integrants that is typical for cell lines, means that many colonies must be screened to find targeted recombinants—a substantial barrier to routine targeting. Promoter trap strategies can give a significantly better enrichment in cell lines when careful attention is paid to matching the expression level of the selectable marker to that of the target gene and to applying the correct stringency of selection (32, 33). Additionally, there is often uncertainty as to the number of genes to be targeted because most cell lines are not perfect diploids. Thus, obtaining targeted recombinants in cell lines currently requires significant up-front characterization or extensive downstream screening.

The avian leukosis virus-induced chicken B cell line DT40 deserves special mention. DT40 cells have slightly elevated levels of HR and much reduced levels of random integration, which together yield a targeting ratio of 10–100% without the need for selection tricks (34). The ease of targeting in DT40 cells has made them an increasingly important model system for studying vertebrate cell biology and has contributed enormously to our knowledge of HR (5). Although DT40 cells have specialized

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Abbreviations: TFO, triplex-forming oligonucleotide; NHEJ, nonhomologous end joining; HR, homologous recombination; ES, embryonic stem; ATM, ataxia telangiectasia mutated; PARP, poly (ADP-ribose) polymerase.

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Table 1. Stimulation of HR by overexpression of *RAD51* and *RAD52* cDNAs

Gene	Promoter	Cell line	Levels*	Substrate	Stimulation‖	Ref.
Hs*RAD52*	CMV	Monkey (FSH2)	ND	*LacZ*†	3–5	45
Cg*RAD51*	SV40	Hamster (SPD8)	1.6–1.8	*HPRT*‡	2–3	58
Cg*RAD51*	SV40	Hamster (AA8)	2–3	*LacZ* or *neo*†	15–35	59
Hs*RAD51*	CMV	Monkey (FSH2)	ND	*LacZ*†	3–4	60
Mm*RAD51*	CMV	Hamster (DRA-10)	~2	*neo*§	2–5	61
Hs*RAD51*	CMV	Human (HT1080)	4	*HPRT*¶	2–4	62

Hs, human; Cg, Chinese hamster; Mm, mouse; CMV, cytomegalovirus promoter; SV40, simian virus 40 early promoter; ND, not determined.

*Numbers indicate fold expression above endogenous gene.

†Intrachromosomal HR between integrated repeats.

‡Intrachromosomal HR between duplicated segments of endogenous *HPRT*.

§Break-induced and spontaneous intrachromosomal HR between integrated repeats.

¶Gene targeting at *HPRT*.

‖Numbers indicate fold stimulation relative to normal cells.

features not present in mammalian cells, they yield results on HR that match those obtained with ES cells and other cell lines. The very existence of this cell line suggests that it should be possible to modify the intrinsic recombination properties of cells to make them more suitable for gene targeting.

Ideally, any strategy for improving gene targeting should be applicable to all vertebrate cell lines and require no prior modification of the cells. It is unlikely that there is a single "magic bullet" that will dramatically improve targeting efficiency in all cells. More probably, individual treatments will yield small improvements that will need to be combined to significantly improve targeting efficiency. Components of such a targeting "kit" might include genes for transient expression of recombination proteins, protocols for modification of targeting vectors, and methods for selectively damaging the target gene to stimulate local HR. Treatments that increase HR or decrease random integration are equally desirable. Below we discuss current research in each of these areas.

Transient Alteration of Gene Expression

HR. Candidate genes for enhancing gene targeting might logically be found among those genes involved in HR. The *RAD52* epistasis group in *S. cerevisiae* comprises several genes for HR in mitotic cells. These can be divided into two families based on the biochemical properties of their encoded proteins (9). One group—*RAD51*, *RAD52*, *RAD54*, *RAD55*, and *RAD57*—encodes proteins involved in the reactions of strand transfer; the other—*MRE11*, *RAD50*, and *XRS2*—encodes proteins required for nuclease activity. At the most simplistic level, Mre11p, Rad50p, and Xrs2p (the designation p indicates the protein product of a gene) control the modification of a broken DNA end to create a single-stranded tail, which then becomes the substrate for strand transfer by Rad51p, Rad52p, Rad54p, Rad55p, and Rad57p. Homologues for these genes have been identified in vertebrate cells, and their effects on HR have been studied in deficient cells.

Rad52p binds single-stranded DNA at its terminus (35), cooperating with RPA, the eukaryotic single-strand binding protein, to prepare the strand for efficient binding by Rad51p before strand invasion (36–42). A single homologue of *RAD52* was identified in vertebrate cells and knocked out in mouse ES cells (43) and chicken DT40 cells (44). The deficient cells show a 2-fold reduction in gene targeting (43, 44), a surprisingly mild phenotype given the essential role of *RAD52* in all HR events in *S. cerevisiae* (9). This finding may indicate a functional redundancy for *RAD52* in vertebrate cells (43). Stable overexpression of human *RAD52* in monkey cells stimulates intrachromosomal HR 3- to 5-fold (45) (Table 1). Thus, *RAD52* is a candidate gene for enhancing targeted HR.

Rad51p, like its bacterial homologue RecAp, oligomerizes on single-stranded DNA to form filaments that catalyze strand exchange (46, 47). Rad51p forms filaments on either 3′ or 5′ single-strand DNA tails and promotes strand invasion without regard for polarity (42, 48). Two other RecAp relatives, Rad55p and Rad57p, help load Rad51p onto single strands (49). Seven vertebrate homologues of *RAD51* have been identified: *RAD51*, *DMC1*, *XRCC2*, *XRCC3*, *RAD51B* (*RAD51L1/hREC2*), *RAD51C* (*RAD51L2*), and *RAD51D* (*RAD51L3*). Homozygous knockouts of *RAD51* (50, 51) and *RAD51B* (52) cause embryonic lethality in mice, and deficient ES cell lines have not been obtained. Conditional *RAD51* mutants in DT40 cells accumulate breaks and die when *RAD51* expression is repressed (53). In contrast to the results in mice, a knockout of *RAD51B* in DT40 cells is viable (54), as are hamster cells with defects in *XRCC2* or *XRCC3* (24). For all three deficiencies, HR is severely impaired. In *RAD51B*-deficient DT40 cells gene targeting is reduced more than 50-fold (54), whereas in *XRCC2*- and *XRCC3*-deficient hamster cells intrachromosomal HR is decreased about 100-fold (55–57). Stable overexpression of *RAD51* stimulates spontaneous HR (58–61), break-induced intrachromosomal HR (61), and gene targeting (62) mostly 2- to 5-fold (Table 1). In addition, in a panel of immortal human cells lines *RAD51* mRNA levels and intrachromosomal HR were elevated to the same extent—3- to 7-fold and 4- to 5-fold, respectively—relative to primary cells (63). Thus, *RAD51* and its family members are also candidate genes for enhancing targeted recombination by overexpression.

A human ubiquitin-like protein, Ubl1p, which is expressed in many human tissues, associates with Rad51p and Rad52p in a yeast two-hybrid system (64). Overexpression of Ubl1p, or of a mutant Ubl1p that cannot be conjugated to its target proteins, inhibits break-induced intrachromosomal HR in hamster cells (65). Although the relationship between Ubl1p and HR is unclear, interference with Ubl1p expression might be expected to stimulate gene targeting.

Rad54p, which belongs to the SNF2/SWI2 family of DNA-dependent ATPases implicated in remodeling chromatin structure (66), appears to act on the duplex DNA target to stimulate Rad51p-mediated strand exchange (67). One of the two homologues of *RAD54* (68) has been knocked out in mouse ES cells (69) and DT40 cells (70) to create viable *RAD54*-deficient cells. Targeted HR is reduced 5- to 10-fold in *RAD54*-deficient mouse ES cells (69) and 100-fold in deficient DT40 cells (70). Thus, Rad54p is critical for targeted HR and a prime candidate for stimulation of gene targeting by overexpression.

The RecQ family of DNA helicases, which includes the genes mutated in Bloom's syndrome, *BLM* (71), and Werner's syndrome, *WRN* (72), offers additional promising candidates. The hallmark feature of Bloom's syndrome is hyperrecombination between sister chromatids and homologous chromosomes (73).

Knockout of the yeast *SGS1* gene, which encodes a RecQ helicase, produces a similar hyperrecombination phenotype (74), which can be suppressed by introducing the *BLM* or *WRN* gene (75). Blmp binds to Holliday junctions—intermediates in HR—and promotes branch migration, perhaps as a way of disrupting Holliday intermediates that arise inappropriately at stalled replication forks (76). *BLM*-deficient chicken DT40 cells have elevated rates of sister-chromatid exchange and enhanced gene targeting (77). These effects are clearly due to HR because they are eliminated by the simultaneous disruption of *RAD54* (77). Transient interference with Blmp might be expected to stimulate gene targeting.

Three tumor suppressor genes, *BRCA1*, *BRCA2*, and *p53*, which have no homologues in yeast, encode proteins that bind to Rad51p (78–82). *BRCA1*- or *BRCA2*-knockout mice die during embryonic development (83–86), whereas p53 knockouts appear normal but are prone to tumor formation (87). *BRCA1*-knockout ES cells display not only decreased levels of intrachromosomal HR and gene targeting, but also increased frequencies of random integration (88, 89). Thus, absence of Brca1p worsens the targeting ratio by simultaneously decreasing the numerator and increasing the denominator. Brca1p interacts with the Mre11p–Rad50p complex (90) in addition to Rad51p, which may account for its influence on both HR and NHEJ (see below). Overexpression of Brca1p might be expected to enhance the efficiency of gene targeting.

In contrast to Brca1p deficiency, loss of p53 stimulates extrachromosomal and intrachromosomal HR 10- to 100-fold or more (91–95). Mutational analysis suggests that p53 affects HR independent of its role in G_1/S cell cycle checkpoint control (96, 97). p53 binds to heteroduplexes at sites of Rad51p-mediated strand exchanges (82, 98) and may be sensitive to mismatches (99). Expression of a dominant mutant of p53 or of simian virus 40 large T antigen, which binds p53, in cells with normal levels of p53 renders them phenotypically p53 deficient and stimulates HR (91, 93). These results suggest that p53 acts as a brake on HR and thus, that temporary interference with p53 might stimulate gene targeting.

Mre11p, Rad50p, and Xrs2p form a complex that is involved in myriad processes in yeast, including HR and NHEJ (9, 100). In the vertebrate complex Nbs1p (p95) is found in place of Xrs2p (101–103). Homozygous *NBS1* deficiency in humans causes Nijmegen breakage syndrome, which is characterized by sensitivity to ionizing radiation and increased cancer incidence (104, 105). Knockout of *RAD50* causes early embryonic lethality in mice (106) and neither homozygous *RAD50*-deficient (106) nor *MRE11*-deficient (107) ES cells are viable. Conditional null mutants of *MRE11* in chicken DT40 cells accumulate breaks and ultimately die upon repression of *MRE11* (108). Given the minimal effects of *MRE11*, *RAD50*, or *XRS2* nulls on HR between yeast chromosomes (109) and the involvement of Mre11p in NHEJ (110, 111), interference with the complex might be expected to improve the targeting ratio. In *MRE11*-deficient DT40 cells, however, gene targeting itself is reduced (108), suggesting that overexpression of members of the complex might be more promising. At present a rational choice between interference and overexpression is difficult.

NHEJ. Candidate genes for depressing random integration to improve the targeting ratio might reasonably be sought among the genes implicated in NHEJ. In addition to the Mre11p/Rad50p/Nbs1p complex, the three subunits of the DNA-dependent protein kinase, Ku70p, Ku80p, and DNA-PKcs, along with Xrcc4p and ligase IV, are involved in NHEJ in vertebrate cells. DNA-dependent protein kinase and the Mre11p/Rad50p/Nbs1p complex may mediate subpathways of NHEJ (108, 112). The DNA-dependent protein kinase pathway has been studied most extensively in the context of V(D)J recombination in the immune system where programmed breaks are rejoined during B and T cell development. Mouse cells deficient in any component of the DNA-dependent protein kinase pathway do not support V(D)J recombination (113–118). Rare coding joints formed in Ku80p-deficient mice exhibited an increase in short sequence homologies (119), similar to the microhomologies associated with Mre11-directed NHEJ (111).

Break-induced intrachromosomal HR and gene targeting are normal in Chinese hamster cells lacking Ku80p (120). However, Ku80p-deficient cells are very sensitive to restriction enzyme-induced chromosome breaks (121, 122), and the rejoining of I-SceI-induced breaks is depressed by 2 orders of magnitude (120). In contrast, the joining of transfected DNA ends is little affected by the absence of Ku80p (123–125) or Xrcc4p (125), although the spectrum of joints is altered (124, 125), consistent with a second mechanism for NHEJ. Because ligase IV is unstable in the absence of Xrcc4p (126), *LIGIV*-deficient cells would be expected to have the same phenotype as *XRCC4*-deficient cells. Finally, Fanconi anemia cells have a deficiency in a DNA end-joining process that appears to be distinct from the DNA-dependent protein kinase pathway for NHEJ (127).

Remarkably, random integration is the same in normal and Ku80p-deficient hamster cells (120), suggesting that it does not use the DNA-dependent protein kinase pathway for NHEJ or that a second mechanism can substitute efficiently. Consistent with a second mechanism is the observation that random integrants are characterized by more junctional homology than is common for NHEJ (27). Observations on plasmid integration in Ku80p-deficient cells also implicate an additional mechanism for random integration that becomes saturated at high DNA concentrations (128, 129). At low DNA concentrations little difference is observed between wild-type and Ku80p-deficient cells; however, at high DNA concentrations random integration is 5- to 10-fold lower in Ku80p-deficient cells (128, 129).

Clearly, we need more information on the genetic requirements for random integration to design a rational strategy for decreasing it. DT40 cells, with their low frequency of random integration (34), might furnish clues in their expressed levels of NHEJ proteins and would provide a cellular assay for testing specific genes.

Signaling Pathways. In addition to proteins directly involved in HR and NHEJ, the targeting ratio might be manipulated via proteins in the pathways that signal DNA damage. The *ATM* gene, which is defective in the human disease ataxia telangiectasia, encodes a protein kinase that activates multiple signaling pathways in response to DNA double-strand breaks (130). Identified targets of ATM (ataxia telangiectasia mutated) kinase-dependent phosphorylation include Rad51p (131), Blmp (132), Brca1p (133), Brca1p-associated protein CtIP (134), Nbs1p (135), and p53 (136). In ATM kinase-deficient mice intrachromosomal HR is elevated about 2-fold (137). In ATM kinase-deficient human cells intrachromosomal HR and plasmid HR are increased about 100-fold (138, 139) and 10-fold (138–140), respectively. Gene targeting, however, is slightly reduced in *ATM* knockouts in DT40 cells (141). From these results it is unclear whether ATM kinase should be overexpressed or inhibited to improve the targeting ratio. It may be that ATM kinase is too far upstream to be effective and that better targets would be found in branches of the downstream signaling pathways.

The ATM kinase activates the c-Abl tyrosine kinase by phosphorylation (142). Activated c-Abl kinase phosphorylates Rad51p, enhancing its association with Rad52p (143) and also phosphorylates DNA-PKcs, dissociating the DNA-PKcs/Ku complex (144). These effects are in the direction expected to improve the targeting ratio; however, phosphorylation of Rad51p also reportedly inhibits its function in strand exchange (145). It is unclear whether manipulation of this signaling

pathway by transient expression of an activated form of c-Abl kinase or by interference with its expression would be the better choice for improving the targeting ratio.

Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme that, when activated by DNA strand breaks, adds ADP-ribose polymers to target proteins (146). PARP knockout mice (147, 148) and PARP-deficient cell lines (149) are viable but more sensitive to gamma irradiation. Overexpression of PARP suppresses sister-chromatid exchanges (150), which are caused by HR (151), whereas overexpression of a dominant-negative PARP increases them (152), as does inhibition of PARP by 3-aminobenzamide (153). Inhibition of PARP increases extrachromosomal and intrachromosomal HR up to 5-fold (154–156) and decreases random integration up to about 100-fold (154, 157, 158). Inhibition of PARP improves the targeting ratio up to 100-fold, when the DNA is introduced by calcium phosphate coprecipitation, but curiously, there is no improvement with electroporation (159). These results suggest that the targeting ratio can be improved under certain conditions of transfection.

Summary. Collectively, current research into HR in vertebrate cells offers several promising candidate genes, whose transiently altered expression levels might reasonably be expected to improve the efficiency of gene targeting. Short-term modulation of expression levels is critical because sustained changes in these genes are likely to be deleterious to the genome. Overexpression of Rad52p, Rad51p (and other family members), Rad54p, and Brca1p and interference with the expression of Ubl1p, Blmp, and p53 might be expected to improve gene targeting. For members of the Mre11p/Rad50p/Nbs1p complex it is unclear at present whether interference or overexpression would be a better choice. It is disappointing that we know so little about the mechanism of random integration, the major barrier to gene targeting in vertebrate cells. In the absence of knowledge, a sensible approach might be to try to disable both suspected subpathways of NHEJ by interfering with expression of components of the Mre11p/Rad50p/Nbs1p complex and of the DNA-dependent protein kinase pathway (Ku70p, Ku80p, DNA-PKcs, Xrcc4p, and ligase IV). The correlation between PARP activity and random integration is intriguing, but the two have not been mechanistically linked as yet. Our understanding of the DNA repair signaling pathways is still too rudimentary to present obvious targets, but ongoing research promises to delineate these pathways in detail.

Modification of Targeting Vectors

Two basic vector designs—replacement vectors (ends-out) and insertion vectors (ends-in)—are used for gene targeting. Replacement vectors, which offer the possibility for one-step modification of the target locus, are far more common. The specific advantages of each type of vector and the enormous power of site-specific recombination systems such as Cre/Lox and FLP/FRT for subsequent manipulation of a targeted site are reviewed elsewhere (13, 159). Gene targeting using either design has a common requirement for shared homology between the vector and the target locus, with the frequency of targeting increasing with longer homology up to at least 10 kb (160–163). Base pair heterologies between vector and target DNAs can decrease the frequency of gene targeting (161, 164, 165), leading to a preference for isogenic DNA in vector construction. By contrast, blocks of heterologous DNA within the vector or at its ends minimally affect targeting frequency (166, 167).

Within this general framework, are there vector modifications that might provide a better substrate for gene targeting and/or a worse substrate for random integration? Addition of dideoxynucleotides to the 3′ ends of linear DNA decreases end joining about 5-fold relative to extrachromosomal HR (168); however, it does not reduce random integration and was not tried in targeting experiments. This same modification enhances targeted HR in *Dichtyostelium discoideum* in two ways without altering the frequency of random integration (169). First, the modification prevents ligation of transfected DNA and, thereby, reduces integration of multiple copies at the target locus: a common problem in *Dichtyostelium*. Second, it has the unanticipated effect of increasing the potency of positive-negative selection, apparently by protecting the negatively selectable marker—at the end of the construct—from degradation. A similar effect in cell lines might enhance the usefulness of positive-negative selection.

A second strategy for improving targeting efficiency is to strip the vector ends to leave single-strand tails a couple of hundred nucleotides long (170). This treatment improves the targeting ratio 5- to 10-fold, independently of whether the vector has 3′ or 5′ single-strand tails (170). A lack of effect of tail polarity fits with the way Rad51p forms filaments (42, 48). The basis for the improved targeting ratio is unclear, but such a tailed substrate might bypass the usual decision-making machinery (perhaps the Mre11p/Rad50p/Nbs1p complex) and preferentially enter the HR pathway by directly binding Rad51p and its helpers. A deeper understanding of the basis for this effect would be useful for further vector improvements.

One of the most profound effects on the targeting ratio, and one of the least understood, is the method of delivery of the vector DNA. Early experiments using microinjection yielded about one targeted recombinant per 10–20 random integrants (2, 171). The specialized equipment and expertise required for microinjection was rapidly replaced by mass delivery methods, with electroporation currently the most common choice. Simplicity, however, comes at the price of decreased targeting efficiency. A systematic study of the targeting ratio at the *APRT* locus in Chinese hamster ovary cells as a function of transfection method is shown in Table 2. The targeting ratio varied from 1:15 for microinjection to 1:370,000 for Fugene-6, with electroporation at 1:2,400 the clear winner among mass delivery methods. Previous head-to-head comparisons of the targeting ratio for electroporation versus calcium phosphate coprecipitation (158, 172) and versus lipofectamine (173) showed the same trends.

Several points are worth noting about these data. (*i*) Random integration tracks reasonably well with transfection efficiency if both the percentage of transfected cells and the intensity of expression are taken into account, suggesting that gene expression and random integration depend on the number of molecules delivered to the cell. (*ii*) For the mass delivery methods gene targeting varies minimally, whereas random integration varies by more than 100-fold, consistent with a copy number dependence for random integration and copy number independence for targeted recombination (3, 31, 179). (*iii*) Site-specific integration, which requires efficient expression of one plasmid and integration of the other, is especially low for electroporation, suggesting that it is the least efficient method for introducing molecules into the cell. The low transfection efficiency and low frequency of random integration with electroporation support this conclusion. Collectively, these observations suggest that an ideal mass delivery method for the best targeting ratio would introduce very few vector molecules into every cell in the population. Of the current methods, electroporation most closely approximates this ideal. The HR machinery also may be overwhelmed by excessive amounts of potential substrates, as suggested by similar frequencies of random integration with microinjection and lipofectamine and frequencies of gene targeting that differ by 4 orders of magnitude. This perspective leads to the counterintuitive idea that the targeting ratio might be improved at lower concentrations of vector DNA.

Table 2. Targeting ratio as a function of method of delivery of vector DNA

Transfection method*	Transfection efficiency†	Random integration‡	Site-specific integration§	Gene targeting¶	Targeting ratio‖
Microinjection	ND	1.2×10^{-1}	ND	8×10^{-3}	1:15
Electroporation	10% (30)	8.7×10^{-4}	2.2×10^{-7}	2.1×10^{-7}	1:2,400
Calcium phosphate	24% (155)	2.5×10^{-2}	1.2×10^{-4}	6.3×10^{-7}	1:40,000
Fugene-6	92% (22)	4.8×10^{-2}	7.2×10^{-4}	1.3×10^{-7}	1:370,000
LipofectAmine	98% (257)	2.2×10^{-1}	1.5×10^{-3}	6.2×10^{-7}	1:350,000

ND, not determined.

*Microinjection (G. N. Proctor, and J.H.W., unpublished work) used vector pAG-7 and cell line CHO-ATS-49tg (174). Mass delivery methods used vector pGS100, cell line RMP41 (175), and published protocols (175–178).

†Transfection efficiency was measured by FACS analysis of cells transfected with plasmid pEYFP-N1 (177). Counting gates were set to include 99% of cells treated in the absence of DNA. Percentages indicate the fraction of treated cells shifted across the counting gate after transfection; numbers in parentheses indicate the mean fluorescence intensity (arbitrary units) of the shifted population.

‡Random integration was measured by selecting for GPT^+ colonies after transfection of *Xho*I-linearized pGS100 (175).

§FLP/FRT-mediated site-specific integration of pGS100 into the *APRT* gene in RMP41 cells was measured after cotransfection of circular pGS100 and pOG44, which expresses the FLP recombinase (175).

¶Targeted HR was measured by selecting for $APRT^+$ colonies after transfection of *Xho*I-linearized pGS100 (175).

‖The targeting ratio is targeted recombinants to random integrants.

Introduction of Gene-Specific Damage

Endonucleases. Surprisingly, the search for homology is not a limiting step for gene targeting in mammalian cells (3, 31, 179), in sharp contrast to yeast (180). Subsequent studies showed that DNA damage, and specifically double-strand breaks, may be rate limiting for HR in mammalian cells. Chromosomal double-strand breaks, introduced by restriction enzymes and rare-cutting endonucleases such as I-*Sce*I and PI-*Sce*I, stimulate intrachromosomal HR (178, 181–184) and gene targeting (185) 10- to 10,000-fold. I-*Sce*I, in particular, which may introduce a unique double-strand break in mammalian genomes at the site of a preintegrated recognition sequence, has proven immensely valuable as a tool to investigate mechanisms of NHEJ and HR (55, 56, 89, 120, 178, 186–191). I-*Sce*I also has been used for repetitive modification of a target locus in mouse ES cells; however, the locus must first be altered to carry the recognition site (192).

The requirement for prior modification means that rare-cutting endonucleases such as I-*Sce*I cannot be used as general tools to stimulate targeted HR, nor can restriction enzymes be used because their sites are all too common. Chimeric nucleases that combine a nonspecific cleavage domain with a specific DNA binding domain (193), in principle, offer a general way to deliver site-specific double-strand breaks to the genome. In *Xenopus* oocytes, a chimeric nuclease composed of the nonspecific cleavage domain from a type IIs restriction enzyme and a DNA-binding domain made up of three zinc fingers, stimulates extrachromosomal HR in a plasmid substrate carrying the cognate sequences for its zinc fingers (194). If zinc finger proteins that have high affinity and sufficient specificity to recognize unique sites in the genome can be developed (195, 196), it may be possible in the future for an investigator to select from a bank of chimeric nucleases one that will cleave a specific gene to stimulate targeted HR.

Triplex-Forming Oligonucleotides (TFOs). TFOs are well-characterized reagents with demonstrated high affinity for DNA and genome-unique specificity. TFOs can deliver a chemical damaging agent to a genomic target to induce site-specific damage (197). TFOs bind with high affinity to polypurine sites in duplex DNA, thereby forming triple-helical DNA structures. The binding specificity of TFOs results from hydrogen bonds formed in the major groove between the TFO and the purine-rich strand of the target duplex (198). High-affinity, statistically unique sites for triplex formation occur about once per kb in vertebrate genomes, and thus, should be present in virtually any gene selected for modification (199). TFOs have been used to inhibit gene-specific transcription (197, 200) and introduce localized mutations in cells and animals (197, 201, 202). Here we focus on TFO-mediated stimulation of HR (Table 3).

Psoralen is commonly used with TFOs because it is stable and innocuous until activated by exposure to UVA light (wavelengths that do not harm cells), at which time it efficiently forms monoadducts and interstrand crosslinks (203). Interstrand crosslinks, in particular, present a formidable challenge to cell viability due to their interference with duplex opening during transcription, replication, recombination, and repair. Interstrand crosslinks are repaired by an intricate interplay of replication, nucleotide excision repair, and HR (204–206). Importantly, psoralen-mediated crosslinks substantially stimulate HR in yeast (207).

TFO-directed psoralen crosslinks stimulate extrachromosomal HR 3- to 25-fold in plasmid-based targets in mammalian cells (208, 209). The first chromosomal target studied is a site in the *APRT* gene in Chinese hamster ovary cells at which psoralen-TFOs form monoadducts exclusively (210, 211). At this site the cognate psoralen-TFO stimulates intrachromosomal HR 3- to 5-fold after UVA irradiation, whereas a control psoralen-TFO irradiated with UVA, the specific psoralen-TFO in the absence

Table 3. Effects of psoralen-modified TFOs on HR in mammalian cells

Cell line	TFO delivery	Adduct	Target	Stimulation¶	Ref.
Human (Jurkat)	Electroporation	Crosslink	*supF**	3	208
Monkey (COS)	Electroporation	Crosslink	*supF*†	25	209
Hamster (AT3-2)	Passive diffusion	Monoadduct	*APRT*‡	3–5	212
Mouse (LTK-)	Microinjection	Crosslink	*TK*§	2,500	213

*Extrachromosomal HR between plasmids.

†Extrachromosomal HR in a plasmid duplication.

‡Intrachromosomal HR between duplicated segments of endogenous *APRT*.

§Intrachromosomal HR between integrated repeats.

¶Numbers indicate fold stimulation relative to untreated cells.

of UVA, and UVA irradiation alone do not affect HR (212). Psoralen-TFOs designed to introduce crosslinks into a chromosomal target increase intrachromosomal HR 2,500-fold when microinjected into nuclei (213). Curiously, this stimulation is independent of UVA irradiation (213). Other studies also report UVA-independent effects of psoralen-TFOs, as well as effects of unmodified TFOs, suggesting that triplex DNA itself may be perceived as damage by the cell (201, 202, 214). Consistent with this possibility, DNA sequences capable of forming intramolecular triplexes stimulate extrachromosomal HR 3- to 5-fold in mammalian cells (215). The molecular details of the cellular repair of triplexes and TFO-psoralen monoadducts and crosslinks are not yet defined.

Clearly, triplex-induced DNA damage can stimulate HR in mammalian cells, but the extent is extremely variable. This variability is likely caused by several factors, including cell type, triplex target site, type of damage, stability of TFOs in cells, HR assay, and TFO delivery method. Of the techniques used to deliver TFOs to cells, microinjection yielded the greatest increases in HR (reminiscent of the results with microinjection of targeting vectors in Table 2), suggesting that TFO uptake and stability may be limiting factors. Further studies of TFO delivery to enhance the frequency of triplex-induced HR are needed.

Conclusions and Perspectives

From the above discussion it is clear that the efficiency of gene targeting is not a fixed quantity. The targeting ratio differs in certain genetic environments and the investigator can manipulate it. Thus, it should be possible to alter cellular DNA metabolism transiently, by overexpressing some proteins and interfering with the expression—or function—of others, to encourage gene targeting and discourage random integration. Potential improvements to vector design have not been fully explored and methods of DNA delivery that enhance the targeting ratio need to be sought. Finally, chimeric nucleases and TFO technology offer hopeful avenues for delivering site-specific damage to the genome as a way to stimulate HR at preselected targets. Incremental improvements in each of these areas might be used in combination for additive (or synergistic) effects.

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