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REVIEW ARTICLE

Recombinational DNA Repair in Cancer and Normal Cells: The Challenge of Functional Analysis

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A major goal of current cancer research is to understand the functional consequences of mutations in recombinational DNA repair genes. The introduction of artificial recombination substrates into living cells has evolved into a powerful tool to perform functional analysis of DNA double strand break (DSB) repair. Here, we review the principles and practice of current plasmid assays with regard to the two major DSB repair pathways, homologous recombination and nonhomologous end-joining. A spectrum of assay types is available to assess repair in a wide variety of cell lines. However, several technical challenges still need to be overcome. Understanding the alterations of DSB repair in cancers will ultimately provide a rational basis for drug design that may selectively sensitize tumor cells to ionizing radiation and chemotherapy, thereby achieving therapeutic gain.

INTRODUCTION

The past few years have seen an explosive increase in the understanding of both the molecular mechanisms and the genetic determinants of recombinational DNA repair. Mutations in genes controlling recombination lead not only to defective repair of DNA double strand breaks (DSBs) and hypersensitivity to ionizing radiation (IR), but also to genomic instability, developmental failure, and carcinogenesis [1, 2]. Particular excitement has been generated by the finding that several cancer susceptibility syndromes result from mutations in recombination-associated genes, including ataxia telangiectasia (*ATM*), Nijmegen breakage syndrome (*NBS1*), AT-Like disorder (*Mre11*), Fanconi Anemia (*FANC* genes), Werner Syndrome (*WRN*), and certain familial breast cancers (*BRCA1/2*) [1, 2]. Sporadic mutations and polymorphisms in a variety of genes involved in recombination have been found not only in normal tissues but also in malignant tumors, including *BRCA1*, *RAD54*, *RAD52*, and *XRCC3* [3, 4]. However, it is largely unknown what the functional significance of these alterations is. There is tremendous interest in a better understanding of the intricate protein networks in which these and other gene products cooperate. Undoubtedly, this knowledge will ultimately have significant implications for cancer prevention and treatment.

The task of characterizing the in vivo repair phenotype that results from any given spectrum of mutations in normal and malignant human cells is challenging. Several assays are available, including the determination of cellular sensitivity to genotoxic agents, cytological evaluation of repair-associated processes and molecular analysis of DNA repair products. Our laboratory has focused on the derivation and application of DNA plasmid assays, which have evolved as a powerful tool for the study of recombination in living cells. Here, we review the principles and practice of current plasmid assays with regard to the two major double strand break repair pathways, homologous recombination (HR), and nonhomologous end-joining (NHEJ). Due to space limitations, we are only able to consider a small number of studies and data sets. For details on mechanisms and genetic determinants of HR and NHEJ, the reader is referred to excellent recent review articles [1, 4, 5] (*including this issue of JBB*, *DNA Damage, Repair, and Diseases*).

HOMOLOGOUS RECOMBINATION

Chromosomal DSB repair assays utilizing the I-SceI endonuclease

The repair of a DSB by HR requires an undamaged template molecule that contains a homologous DNA sequence. Such a template can be provided by the sister chromatid, the homologous chromosome, or an adjacent repetitive sequence on the same chromosome. Specifically designed DNA plasmid substrates typically model homology-directed DSB repair by utilizing tandem repeats of a bacterial antibiotic resistance gene. Commonly, the 18 base pair recognition sequence for the rare-cutting I-SceI endonuclease is introduced into one gene copy, thereby inactivating gene function (for review [6]). The second copy is made inactive by other means. The repair substrate is stably integrated into the cell's genome (Figure 1a). After the introduction of a break at the I-SceI site, only a homology-mediated event can reconstitute the gene and confer cellular resistance to a selection antibiotic in cell tissue culture. The design of the tandem repeat and the I-SceI insertion site can be modified to allow for the

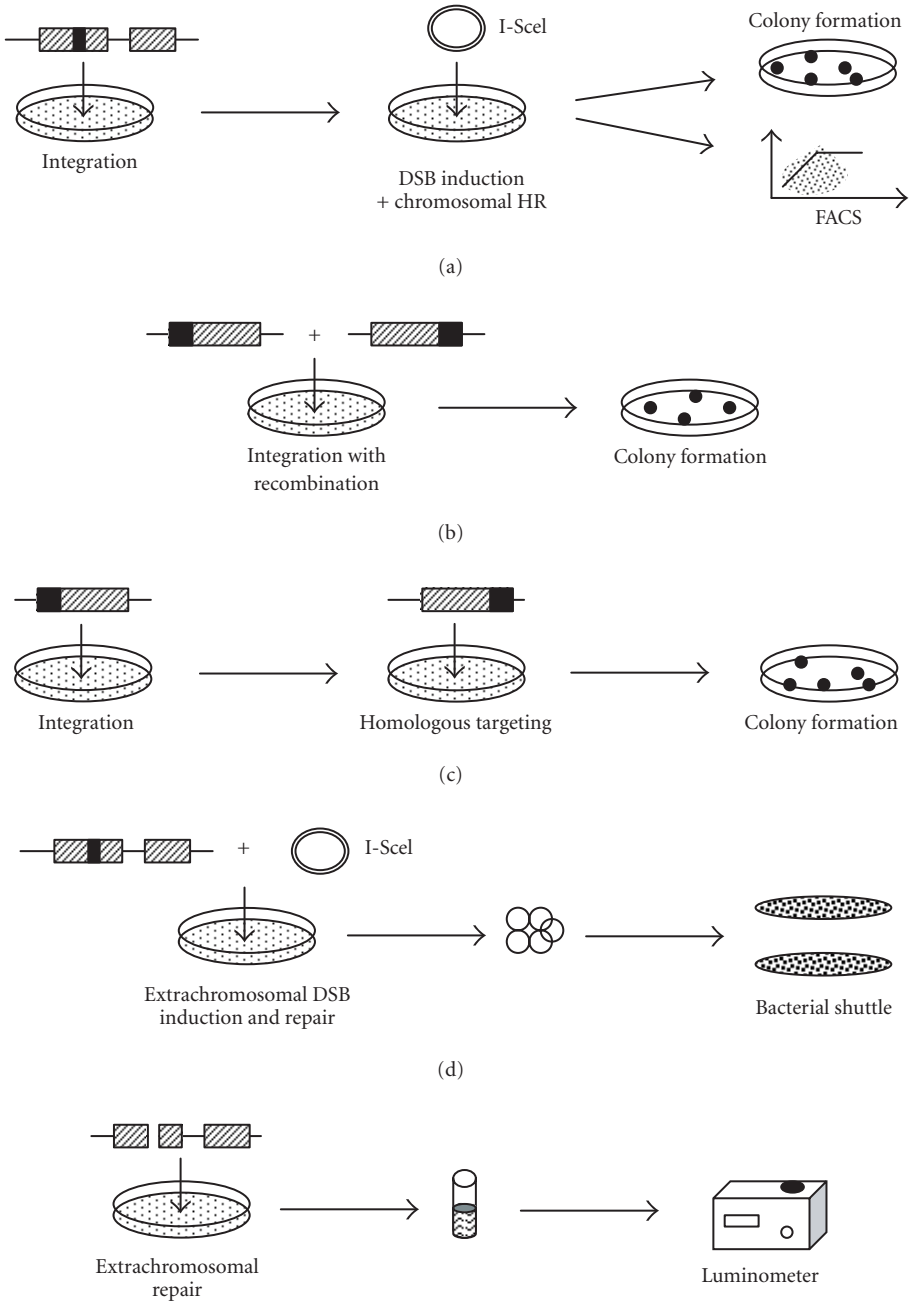

Figure 1. Illustration of the principles of measuring HR with plasmid assays. (a) Chromosomal DSB repair assay [14, 15]. A linearized plasmid substrate containing mutated resistance or marker genes is transfected and stable chromosomal integrants are selected, for example, using a puromycin resistance gene. Single-cell derived or pooled populations are amplified, and the integrated plasmid is characterized. Single or multiple copy integrants can be processed. The I-SceI expression vector is transiently transfected and usually 48 hours are allowed for the generation and homology-directed repair of I-SceI breaks within the integrated substrate (black insertion). The HR frequency can be assessed by selecting for the recombined antibiotic resistance gene in a colony formation assay (eg, *GPT*), or by assaying the marker gene product such as the green fluorescent protein (GFP) via flow cytometry (FACS). (b) Integration-associated HR [18]. The linearized substrates pΔ2 and pΔ3, each carrying a mutated *GPT* gene copy (black boxes, gene deletions), are cotransfected. HR before and during plasmid integration leads to the reconstitution of the resistance gene. Drug selection is used for isolation of stable recombinants in a colony formation assay. (c) Homologous gene targeting [29]. Linearized pΔ2puro is stably integrated using puromycin. Transient transfection of pΔ3 allows for HR between the chromosomal *GPT* gene copy and the extrachromosomal *GPT*. The reconstituted *GPT* gene is selected for using colony formation. (d) Episomal HR with replication [30]. An episomally replicating recombination substrate carrying mutated tandem repeats of a *CAT* reporter is transiently transfected together with an I-SceI expression vector. Breaks are generated within one *CAT* copy (black insertion) and repaired via HR using a downstream homologous template. After 48 hours, episomal plasmids are extracted and tested for the functional *CAT* gene in a bacterial shuttle vector assay. (e) Extrachromosomal HR. The mutated recombination substrate (*CAT* or *Luciferase* gene) is cut prior to transfection. HR reconstitutes the gene function, which can be measured by assaying cell extracts in a luminometer or scintillation counter.

assessment of genetically distinct subpathways of HR [7, 8]. Modifications of the plasmid substrate have permitted studying of interchromatid and even interchromosomal recombination [7, 9, 10]. Johnson and Jasin [11] recently reviewed the use of the I-SceI system in mammalian cells.

Many laboratories have employed the I-SceI assay, mostly in rodent cell lines [7, 8, 11, 12, 13, 14]. Several groups including our own have found up to 100-fold or more stimulation of spontaneous recombination frequencies upon the expression of the I-SceI enzyme. These studies have established that chromosomal HR can serve as a significant alternative to NHEJ in mammalian cells. The I-SceI assay has provided insight into the genetic determinants of HR. Reduced repair of I-SceI breaks has been observed in the absence of functional *RAD51*, *RAD54*, XRCC2, XRCC3, BRCA1, and BRCA2, and these findings have correlated with increased cellular sensitivity to IR [7, 8, 14, 15, 16, 17]. Thus, the repair of I-SceI breaks is at least partially carried out by the same protein network that is involved in the repair of radiation-induced DNA damage.

The ATM and p53 gene products, which are central proteins in the DNA damage response pathway, have been reported by us and others as being involved in the regulation of HR [18, 19, 20, 21, 22]. The *P53* gene has been observed to suppress spontaneous HR by at least one order of magnitude in a wide variety of rodent and human cell lines; and this could be a means by which p53 maintains genomic stability [23]. Mutation of *ATM*, which is upstream of *p53*, led to increased and error-prone HR (see below, section Plasmid assays containing extrachromosomal components) [18]. However, it has not yet been established whether and how *ATM* and *p53* influence the repair of the I-SceI breaks.

Several important technical caveats should be considered when utilizing the I-SceI system (Figure 1a). Firstly, there is usually substantial variation in the induction of recombination among several single-cell derived clones, implying that the random integration site can have considerable influence on recombination activity. One way of obtaining a representative sample of the cell population under study is to pool a large number of colonies, which contain plasmids integrated at various random sites in the genome. Secondly, in many situations, extremely large amounts of I-SceI expression vector, that is, up to 100 μg, need to be transfected for the effect to be seen. One possible explanation is that simple religation of I-SceI break ends is the dominating activity and that therefore serial DSBs have to be induced to eventually trigger an HR event. Thirdly, spontaneous HR frequencies can be substantial and thus mask any DSB-induced recombination activity. However, it can be useful to isolate a clone that exhibits a significant spontaneous HR frequency and an at least 10-fold induction after the expression of I-SceI nuclease. This would allow for parallel evaluation of spontaneous and break-induced HR, which potentially have different genetic determinants. Lastly, it is important to stress that the experiment typically spans two or more months involving several rounds of transfection and colony formation. Therefore, it is favorable to develop shorter assays that make use of recombination markers such as the green fluorescent protein (GFP), which will abrogate the need for one round of colony formation in difficult to grow cell lines [14].

Cell line and break type limitations

From the data obtained with the I-SceI repair system, arises the question whether this assay should be considered the "gold standard" to assess proficiency in homology-mediated DSB repair. This assay provides an important model system in cell lines that are characterized by rapid and unlimited growth in tissue culture, by high transfection efficiencies (eg, > 10%) and sufficient colony forming abilities (eg, > 5%). However, these requirements are only met in a selected number of cell lines including immortal lines such as Chinese hamster ovary cells or murine embryonic stem (ES) cells. It is important to recognize that immortal cell lines typically do not possess true functional wild-type p53 status, including ES cells [24, 25, 26]. However, with the loss of p53, the regulation of HR is expected to be relaxed or disturbed [20, 24]. This likely affects the functional analysis of other proteins involved in HR. In most cell lines with wild-type p53 status, successful application of colony formation assays to measure HR is difficult to accomplish because of p53's growth-inhibitory effects. Exceptions may include some tumor lines, for example, MCF-7 [20], and immortal lymphoblastoid lines [27, 28]. Typical limitations to the utilization of complex repair assays such as the I-SceI system are displayed in Table 1.

Induction of chromosomal breaks by the I-SceI endonuclease generates a 4 bp staggered cut that leaves free 3′ hydroxy overhangs, which can be directly religated. As discussed, the proficiency to repair this break type via HR correlates with the resistance to IR. However, IR creates far more complex chromosomal break ends than endonucleases, and it is therefore likely that several protein activities that participate in the repair of radiation-induced chromosomal breaks can be missed by an I-SceI type assay. The technical challenge, therefore, is to design assays that employ ends resembling radiation type damage more closely, and this could include cohesive but noncompatible cut sites, blunt ended cuts, and dephosphorylated double-stranded ends. Consequentially, such an approach would involve variably cut extrachromosomal repair substrates, as no alternative to I-SceI generated chromosomal breaks is yet available.

Plasmid assays containing extrachromosomal components

The role of ATM for extrachromosomal and integration-associated HR was investigated in our laboratory by Luo et al (Figure 1b) [18]. This assay studies intermolecular HR between two plasmids, pΔ2 and pΔ3, which contain a 5′ deletion and a 3′ deletion in the bacterial *gpt* gene, respectively. The gene arrangements are illustrated in Figure 2. The HR and restoration of the *gpt* gene following the cotransfection of the plasmids resulted in a resistance to XHATM in a colony formation assay. When the plasmids were cleaved at a distance from the gene, the HR frequency was 27-fold higher in AT fibroblasts than in normal human fibroblasts. However,

Table 1. Typical limitations to the use of I-SceI based plasmid assays for assessment of chromosomal DSB repair.

Limitations	Cell type
Limited life span	Primary MEFs Primary human fibroblasts
Poor tissue culture features (limited colony formation, growth, and transfection efficiency)	Primary MEFs (especially BRCA1/2 knock-outs) Primary human fibroblasts (especially NBS1) Many tumor lines (eg, Capan-1)
Alteration of wild-type p53 function	Immortalized MEFs Mouse ES cells SV-40 transformed human fibroblasts Most human tumor lines
Apoptosis proficiency	Lymphoblastoid lines

MEF: mouse embryonic fibroblasts.

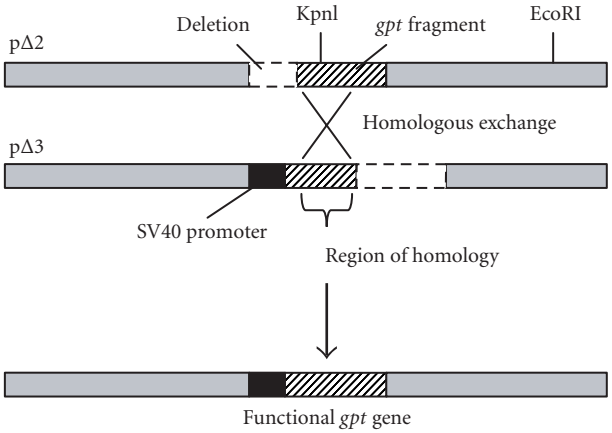

Figure 2. Illustration of integration-associated HR (corresponding to Figure 1b). Plasmid substrates pΔ2 and pΔ3 contain nonoverlapping 5′ and 3′ deletions, respectively. There is an area of shared homology of approximately 400 bp. Experiments were carried out with cleavage at a distance from the gene, that is, EcoRI, or within the gene, that is, KpnI. See [18] for details. Homology-mediated recombination and *gpt* gene restoration can occur before, during, or shortly after chromosomal plasmid integration. Cellular resistance to XHATM is selected for after 48 hours.

upon cleavage within the *gpt* gene, the frequencies differed only by 3-fold. One conclusion derived from these data was that if the DNA termini are within or close to the recombination substrate, then the high frequency of extrachromosomal recombination in AT cells is offset by mis-repair of the recombining sequences.

Figure 1c illustrates an assay modification, which utilizes stable chromosomal integration of pΔ2puro at a random site, transient transfection of pΔ3 and selection of *gpt* recombinants. Using this targeting principle, we could show that BRCA2- and BRCA1-deficient tumor cells are defective in homology-mediated recombination between a chromosomal and an extrachromosomal substrate by one order of magnitude ([29] and unpublished data from our lab). The results of this assay are consistent with other reports showing reduced homologous gene targeting in cells lacking BRCA2 or BRCA1 [16, 17].

Purely extrachromosomal HR, that is, in the absence of any integration step, can be assessed by employing episomal shuttle vectors (Figure 1d). Our data suggest that BRCA2 maintains its stimulatory effect on HR in cancer cells in this somewhat less physiologic assay setting [29]. In contrast, absence of murine p53 has no impact on regulation of HR in extrachromosomal plasmid substrates, in striking difference to the suppression of chromosomal HR [30]. Figure 1e illustrates a rapid plasmid reactivation assay which employs *CAT* or *Luciferase* reporters; however, it is unclear what the genetic determinants of this endpoint are.

Translational research in the postgenome era may ultimately involve routine characterization of repair phenotypes in primary fibroblasts or tumor cells directly taken from patients with known genetic profiles. Universally applicable plasmid assays that partially or entirely contain extrachromosomal components could provide a means to assess recombinational repair in cells that have limited growth characteristics, and to assess the repair of various break types. However, a significant problem to be addressed involves the physiological limits that extrachromosomal repair substrates possess. For example, extrachromosomal DNA is easily subjected to nucleolytic attack and is removed from chromatin regulation processes. Therefore, it becomes critical to correlate the results of extrachromosomal assays with those of I-SceI-based assays in defined model systems and to characterize the genetic determinants of extrachromosomal DSB repair. At this stage of research, it appears that purely extrachromosomal HR proceeds independently of p53, at least in murine fibroblasts [30]; thus necessitating the use of integration-linked assays for analysis of p53-dependent HR (Figures 1b, 1c, and 2). Alternatively, assays utilizing mutated SV40 genomes can provide an important alternative to plasmid systems for assessment of chromatin-associated repair mechanisms [19, 21, 23].

NONHOMOLOGOUS END-JOINING

Religation versus illegitimate rejoining

Nonhomologous end-joining of two double-stranded DNA ends does not require an undamaged partner and does not rely on extensive homologies between the recombining ends. Sometimes, NHEJ can utilize flanking microhomologies spanning 2–6 bp. Rejoining of the ends can occur after limited degradation at the termini. Furthermore, in many cases end-joining involves sequence alterations by small deletions, insertions, or inversions. Thus, the repair process itself is error-prone. It is important to realize that restriction enzyme generated DSBs can be repaired by either simple religation or illegitimate rejoining thereby destroying the original sequence. It has been hypothesized that illegitimate rejoining is more likely to occur with plasmid ends that have been generated prior to cell transfection, possibly because the ends are subject to degradation once entering the cell. In contrast, break induction in vivo such as by I-SceI would be more prone to religation. However, our data suggest that illegitimate rejoining of extrachromosomal I-SceI breaks can occur at a high frequency, though it is difficult to assess the contribution of religation because a religated break is indistinguishable to a recognition sequence that has never been cut [30]. Lin et al [13] introduced a chromosomal repair substrate into mouse L cells, which contained a *TK* gene disrupted by an I-SceI site tandem repeat. Generation of DSBs led to the removal of the intervening sequence and precise religation with reconstitution of one I-SceI site in 33–65% of all NHEJ events. This is likely to be an overestimate, since NHEJ not resulting in restoration of the *TK* open reading frame could not be selected for. In contrast to cuts from endonucleases or restriction enzymes, radiation-induced breaks can only be repaired by an illegitimate repair process.

The dilemma of selection

In contrast to HR, it is difficult to envision how NHEJ can reconstitute the function of a reporter gene, which is needed to allow the identification of repair products within a large cell population. When using a chromosomally integrated reporter gene that is inactivated by the insertion of an I-SceI recognition site, NHEJ would have to precisely remove the insert in order to reconstitute gene function. In principle, this can be facilitated by placing regions of microhomology upstream and downstream of the insert. However, such an assay would only score a fraction of NHEJ events. Previously, investigators have grown up 100 or more unselected colonies after induction of I-SceI breaks in an integrated HR substrate (Figure 3a). Physical analysis using Southern blotting and PCR on a clone by clone basis can provide a quantitative estimate of chromosomal NHEJ; however, this is an extremely laborious process. The only available assay type that uses drug selection for NHEJ events involves measuring the frequency of random stable integration of a linearized plasmid substrate (Figure 3b). Cells lacking components of the nonhomologous repair pathway, for example, DNA-PK$_{cs}$, Ku, or XRCC4, display plasmid integration frequencies that are reduced by 10-fold or more ([31] and unpublished data from our lab). However, factors other than break end-joining proficiency may influence plasmid integration, thus making this assay subject to various biases.

As with HR, NHEJ has been historically studied using extrachromosomal substrates. Several assay principles have been employed. Figure 3c illustrates a variation that we have used recently [32]. Circular plasmid molecules were linearized between the viral promoter and the *Luciferase* reporter gene prior to transfection. In linearized plasmids, the *Luciferase* gene cannot be expressed. Only after DSB rejoining with recircularization of the plasmid, the transcription of the reporter gene can proceed. We showed that p53 could enhance NHEJ of DSBs with cohesive ends by 2- to 3-fold in rodent embryonic fibroblasts, but only in the presence of an additional exogenous DNA damage signal. The data suggested that p53 was enhancing DSB rejoining specifically by increasing the ability to reanneal short complementary strands of single-stranded DNA. Using a similar assay principle, Liang and Jasin [33] observed increased degradation of DNA termini in Ku80-deficient cells. However, in cells lacking DNA-PK$_{cs}$, plasmid rejoining proceeded normally. It is therefore not clear as to which degree the pathways involved in nonhomologous repair of radiation-induced DSBs and in the rejoining of extrachromosomal plasmid ends overlap. A more specific way to study the genetic determinants of NHEJ is to utilize Rag1- and Rag2-initiated site-specific V(D)J recombination as a functional endpoint, which tests for the functional presence of the DNA-PK complex, XRCC4, and ligase IV (Figure 3d) [34]. This assay is analogous in design to the HR shuttle vector assay.

In summary, as for HR, a variety of assays are available to assess NHEJ in difficult and easy to grow murine and human cell lines. However, more study is needed to determine which genetic pathways can be studied by any particular system.

TOWARD THERAPEUTIC GAIN

For genotoxic agents used in cancer treatment, a therapeutic gain is defined by a better relation between the killing of tumor and normal cells in a patient. With regard to IR, it is important to realize that in most instances malignant cells are neither more radiosensitive nor more radioresistant than their normal counterparts. In some cases, mutations in central recombination genes within the tumor appear to confer a defect in DSB repair and consequential radiosensitivity, as suggested for BRCA2 [29]. Such a mutation may either be absent in the normal tissues, or be present as heterozygosity but not conferring any repair phenotype, thus offering therapeutic gain upon treatment with DSB-inducing agents.

It is likely that recombinational repair in malignant cells is generally altered compared to normal cells. For example, the *P*53 gene is mutated or inactivated in the majority of human tumors. Consequentially elevated HR may contribute to the increased loss of heterozygosity and chromosomal instability observed in many tumors, though the causal

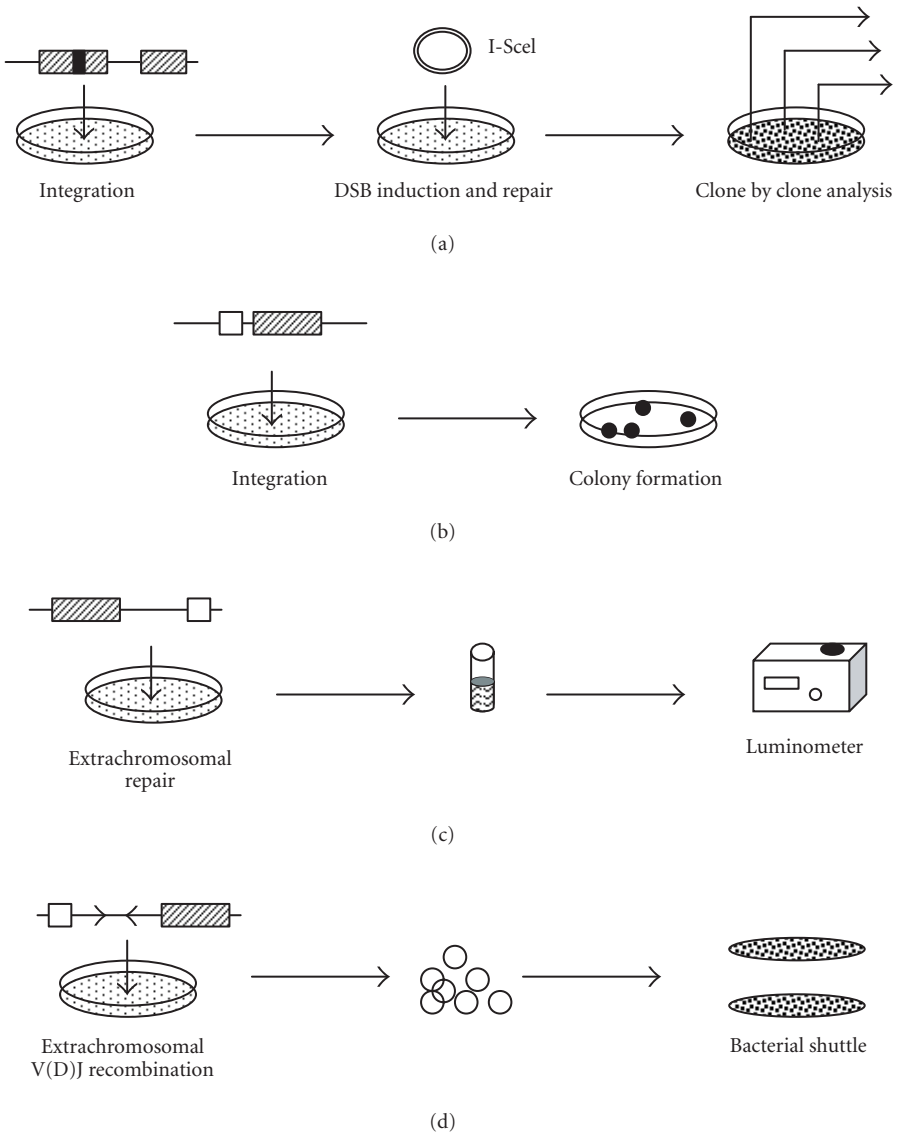

FIGURE 3. Illustration of the principles of measuring NHEJ with plasmid assays. (a) Physical analysis of chromosomal NHEJ. Analogous to Figure 1a, I-SceI breaks are generated in an integrated HR substrate, but cells are plated without selection antibiotic. Colonies are analyzed on a clone by clone basis for an NHEJ product using Southern blot and PCR. Alternatively, PCR of genomic DNA can be used for semiquantitative analysis of NHEJ [16]. (b) Random plasmid integration [29, 31]. The transfection frequency of a linearized reporter plasmid as a measure of NHEJ is scored by selecting for the intact resistance gene, for example, with puromycin. (c) Extrachromosomal rejoining assay [32]. A *Luciferase* expression plasmid is cleaved between the promoter and the reporter gene prior to the transient transfection. In the cell, *Luciferase* can only be expressed following NHEJ and re-circularization of the plasmid. Cell extracts are assayed for *Luciferase* activity. (d) Episomal V(D)J recombination [34]. Analogous to Figure 1d, recombination signal sequences flank a bacterial transcription stop signal upstream of a *CAT* reporter. The plasmid is cotransfected with Rag1/Rag2 expression vectors. The cleavage of the signal sequences followed by site-specific recombination removes the transcription stop signal, so that extracted plasmids can be assessed for *CAT* resistance in a bacterial shuttle vector assay.

relationship has yet to be proven [2]. The loss of cell cycle control in tumor cells results in a larger fraction of cells being in the S and G2 phases of the cell cycle compared to normal cells. Homology-directed DSB repair is thought to be an important pathway in these phases. This is in contrast to the G1 phase, during which NHEJ is likely to dominate because no sister chromatid is available to provide a homologous repair template. Therefore, HR may be commonly elevated and deregulated in cancer cells. This could provide a rationale basis for drug design targeting HR pathways and thereby sensitizing tumors to IR. Therapeutic gain will result especially in relation to non- or slowly proliferating normal tissues, which are largely in the G0 or G1 phase and thus repair mainly via NHEJ. A variety of functional assays including plasmid systems and other means will be needed to characterize DSB repair in cancer and normal cells and to

allow predictions of the outcome of combined treatment approaches. However, as discussed here, several technical challenges with respect to functional analysis in vivo still need to be overcome.

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