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In vitro non-homologous DNA end joining assays—The 20th anniversary

Elzbieta Pastwa^{a,*}, Richard I. Somiari^b, Mariusz Malinowski^c, Stella B. Somiari^d, and Thomas A. Winters^e

^a Department of Molecular Genetics, Medical University of Lodz, Mazowiecka 6/8, 92-215 Lodz, Poland ^b Functional Genomics & Proteomics, ITSI-Biosciences, 633 Napoleon Street, Johnstown, PA 15901, USA ^c Department of Molecular and Medical Biophysics, Medical University of Lodz, Mazowiecka 6/8, 92-215 Lodz, Poland ^d Cell Biology Unit, Windber Research Institute, 620 Seventh Street, Windber, PA 15963, USA ^e Nuclear Medicine Department, Warren Grant Magnuson Clinical Center, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA

Abstract

DNA double-strand breaks (DSBs) are the most serious forms of DNA damage in cells. Unrepaired or misrepaired DSBs account for some of the genetic instabilities that lead to mutations or cell death, and consequently, to cancer predisposition. In human cells non-homologous DNA end joining (NHEJ) is the main repair mechanism of these breaks. Systems for DNA end joining study have been developing during the last 20 years. New assays have some advantages over earlier in vitro DSBs repair assays because they are less time-consuming, allow the use of clinical material and examination of the joining DNA ends produced physiologically in mammalian cells. Proteins involved in NHEJ repair pathway can serve as biomarkers or molecular targets for anticancer drugs. Results of studies on NHEJ in cancer could help to select potent repair inhibitors that may selectively sensitize tumor cells to ionizing radiation (IR) and chemotherapy. Here, we review the principles and practice of in vitro NHEJ assays and provide some insights into the future prospects of this assay in cancer diagnosis and treatment.

Keywords

Anticancer drugs; DNA double-strand breaks; DNA repair; In vitro assays; Non-homologous DNA end joining

1. Introduction

The year 2008 represents the 20th anniversary since the first description of an in vitro non-homologous DNA end joining (NHEJ) assay by Pfeiffer and Vielmetter (1988). Biochemical approaches to study NHEJ are very important, because they can answer many questions that are difficult to answer with genetics. These questions address the mechanism of the repair process, enzymatic activities, and regulation of NHEJ proteins and their physical interaction. Although biochemical studies on proteins associated with NHEJ have always lagged behind studies on at the DNA level, some efforts have been made to identify a single protein required for NHEJ using biochemical purification methods and reconstitution of NHEJ activity. This review summarizes the principles and practice of in vitro biochemical methods for NHEJ.

*Corresponding author. Tel.: +48 42 679 07 22; fax: +48 42 679 08 26. epastwa@csk.umed.lodz.pl (T. Pastwa).

In mammals, DNA double-strand breaks (DSBs), the most lethal form of DNA damage, are mainly repaired by NHEJ (Lieber, 2008; Weterings and Chen, 2008). This pathway does not require homology and can rejoin broken DNA ends directly end-to-end. NHEJ is an error-prone process, with loss or addition of at least one nucleotide at the rejoining site. Simple ligation, which exists during the joining of cohesive or blunt ends, is also classified as a form of NHEJ.

Genetic studies, using radiosensitive mammalian cell lines, that were deficient in DSBs rejoining, have led to the identification of several NHEJ proteins: Ku70, Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}), Artemis, X-ray cross complementation 4 (XRCC4), ligase IV, and recently XRCC4-like factor (XLF, also named Cernunnos) was discovered (Ahnesorg et al., 2006; Buck et al., 2006; Dai et al., 2003; Gu et al., 1997; Lees-Miller et al., 1995; Riballo et al., 1999).

Repair of DSBs by NHEJ has been the centre of attention in recent years because of the recognition that there are individual differences in the capacity to repair DSB's (Chistiakov et al., 2008). Many NHEJ assays can have a broad application in cancer research, including determination of the toxic effect of therapeutic agents and prediction of the recurrence of cancer after treatment (Pavón et al., 2008). Using these assays specific inhibitors of NHEJ pathway can be designed. Such inhibitors can enhance the toxic effect of anticancer drugs and ionizing radiation (IR) in therapies against cancer.

2. Non-homologous DNA end joining mechanism

The basic NHEJ pathway requires the concerted action of the seven proteins mentioned earlier: Ku70, Ku80, DNA-PK_{cs}, Artemis, ligase IV, XRCC4, and XLF. A simple example of NHEJ is shown in Fig. 1.

In the initial step Ku binds to the free DNA ends and thus protects these ends from nucleolytic degradation. Ku is also implicated in alignment, juxtaposition of the two DNA ends in the close proximity (synapsis), ligation and recruitment of factors involved in the processing of DNA ends (Malinowski and Pastwa, 2006).

In the second step, DNA-PK_{cs} is recruited to Ku-bound DNA termini. DNA-PK_{cs} alone has weak protein kinase activity (DeFazio et al., 2002). However, its interaction with Ku/DNA complex stimulates its kinase activity. Activated DNA-PK_{cs} phosphorylates six of the seven known NHEJ proteins: Ku70, Ku80, XRCC4, Artemis, XLF, and DNA-PK_{cs} itself. Only DNA ligase IV is not a target for DNA-PK_{cs} (Shrivastav et al., 2008; van Gent and van der Burg, 2007).

When the structure of the DSB end is simple (cohesive and blunt ends), they can be ligated by a complex of XLF, XRCC4, and DNA ligase IV only (Ahnesorg et al., 2006; Buck et al., 2006). However, when NHEJ involves nucleotide loss or addition at the rejoining site (i.e. radiation-induced DSBs), three types of enzymatic activity are required for repair of such DSBs by the NHEJ pathway: (1) nucleases to remove nucleotides, (2) polymerases to add nucleotides, and (3) ligase to restore the phosphodiester backbone (Lieber, 2008). In the case of nucleases, Artemis becomes an active endonuclease after phosphorylation by DNA-PK_{cs}. In humans, polymerases μ and λ are known to have role in NHEJ.

Finally, a complex of XLF, XRCC4, and ligase IV, which composes the ligase for NHEJ, is required to join the broken DNA ends. However, very recent biochemical analysis showed that XLF is also recruited to DSBs in the initial step of the NHEJ process and that Ku stimulates XLF binding to DNA (Yano et al., 2008).

3. DNA double-strand break type limitations

The hallmark of NHEJ is its ability to deal with the diversity of DSB substrate configurations that arise. The most commonly used in vitro assays for NHEJ is a substrate plasmid DNA linearized with a single restriction enzyme (RE) or with two different restriction enzymes. Although this is an efficient method of linearizing plasmids, the physical structure of the DSBs does not reflect the structure of breaks produced naturally in mammalian cells (Pastwa and Blasiak, 2003). Most naturally occurring DSBs, particularly those produced by IR and some chemotherapeutic agents, are formed by oxidative processes. Oxidatively induced DSBs typically possess far more complex break ends than those produced by endonucleases, and are generally unligatable in the absence of nucleolytic processing (Povirk et al., 2007). Radiation-induced, and other free radical-mediated DSBs, result from oxidative fragmentation of deoxyribose, and typically possess 5'-phosphate and either 3'-phosphate or 3'-phosphoglycolate (PG) ends. These 3'-terminal blocking groups must be removed or modified to produce 3'-hydroxyl ends or DSB repair cannot be completed (Povirk, 2006). Good enzymatic candidates for such 3'-end modifications would seem to be polynucleotide kinase (PNK), which can remove 3'-terminal phosphates (Chappell et al., 2002), human apurinic/apyrimidinic endonuclease (APE1), which removes 3'-PGs from single-strand breaks in DNA (Winters et al., 1992), and tyrosyl-DNA phosphodiesterase (TDP1), which, in conjunction with the sequential action of PNK, can also remove 3'-PG (Interthal et al., 2001; Takashima et al., 2002; Plo et al., 2003). Artemis, acting in a complex with DNA-PK_{cs} as a 53 and 33 overhang endonuclease, also leaves a 3'-hydroxyl, but removes at least one complete nucleotide from the DNA end (Ma et al., 2002). Consequently, several enzyme activities that are required for the repair of IR-induced DSBs can be missed by a traditional RE type assay. Therefore, the technical challenge associated with the in vitro analysis of NHEJ is to design assays that employ DSB ends that more closely resemble naturally occurring (i.e. radiation, oxidative, etc.) DSB damage. Among the many potential structures, this could include 5' dephosphorylated DSB ends, or 3' ends blocked by phosphoglycolate DNA termini. Fortunately, some in vitro NHEJ systems are available to examine the joining of DNA substrates that are physiologically more relevant (Cheong et al., 1998; Gu et al., 1996; Odersky et al., 2002; Pastwa et al., 2001).

4. In vitro non-homologous DNA end joining systems

4.1. The 1st decade

Generally, in vitro NHEJ methods are based on incubation of damaged plasmid DNA with cell extracts as a source of DNA repair activities. Repair products (dimers, trimers, etc.) are identified by agarose gel electrophoresis followed by detection with fluorescent dyes, autoradiography (for ³²P labeled substrate DNA), Southern blotting, or PCR assays (used when NHEJ is inefficient). The typical workflow for in vitro NHEJ analysis is shown in Fig. 2.

NHEJ methods have been evolving during the last 20 years (Table 1). In this year we celebrate the 20th anniversary of developing the first in vitro assay by Pfeiffer and Vielmetter (1988). In this system, the repair efficiency of *Xenopus* egg whole extracts for various combinations of non-matching restriction enzyme-induced DSBs was determined using a Southern blot analysis based assay. Repair fidelity was assessed by sequence analysis of cloned joined products after bacterial transformation and expansion. Based on the results, it was proposed that unknown DNA-binding proteins stabilize DNA ends that were unfavorable for direct ligation.

The same source of extract was introduced by Labhart (1999) to show that Ku heterodimer is also required for the in vitro reaction as it is in vivo in mammalian cells.

The first mammalian in vitro system for NHEJ was described by North et al. (1990). Physical rejoining of RE-induced DSBs in human cell extracts was monitored by Southern analysis and the fidelity of rejoining was determined using a β -glycosidase dependent bacterial mutagenesis assay following bacterial transformation of sensor bacteria with the extract-repaired plasmid. Two years later, the same laboratory developed a rapid and efficient in vitro system for rejoining of DSBs (Fairman et al., 1992). For the first time, ethidium bromide staining was used for the detection of repair products, which minimized the length of time required to perform this NHEJ assay. Additionally, they showed that fractionated nuclear extract required factors other than ligase I, II or III for efficient DSB end joining.

Soon after in Pfeiffer's laboratory, synthetic DNA molecules were employed for the first time as repair substrates in the NHEJ assay they had originally developed in 1988 (Beyert et al., 1994). The strength of this approach is that oligonucleotides can mimic more complex DNA breaks with variable polarity, length and sequence.

Povirk and collaborators (Gu et al., 1996) applied bleomycin-cleaved oligonucleotides to construct NHEJ substrates with 3'-PG ends, similar to those produced by IR. A follow up study (Gu et al., 1998), using human cell extracts, produced interesting results in that 3'-PG DSB ends were not rejoined when compared to Xenopus egg extract.

To meet the technical challenge of designing assays that employ radiation damaged DNA, a unique in vitro NHEJ system was developed by Iliakis and coworkers (Cheong et al., 1998). This concept is based on special preparation of the DNA substrate. Tissue culture cells were embedded in agarose, lysed and treated with RNase and proteinase to obtain genomic DNA. This genomic DNA was then irradiated with X-rays, and this IR-induced damage was repaired by incubation of the agarose-embedded damaged DNA with HeLa extract proteins. DNA repair was measured by asymmetric field inversion gel electrophoresis (AFIGE) (Iliakis and Cheong, 2006). When compared to the in vitro assay based on plasmid DNA, it was determined that the plasmid assay is simpler and it should be chosen for repair measurement if both assays possess the same enzymatic activities required for DSB rejoining. However, these studies led to the conclusion that these two assays showed different enzymatic requirements for DSB rejoining, and therefore are expected to play complementary roles in the characterization of repair factors. The AFIGE assay described above has an important drawback when compared to plasmid based assays: the sequence of the repair joints cannot be determined due to the random nature of the DSB locations produced by IR.

Amongst in vitro systems, the NHEJ assay developed by Baumann and West (1998) is the most popular and widely used. Using this system it was shown for the first time that Ku70, Ku80, DNA-PK α and DNA ligase IV/XRCC4 are involved in human cell-free DNA end joining. In addition, the reaction required inositol hexakisphosphate (Hanakahi et al., 2000; Hanakahi and West, 2002) and polynucleotide kinase for ends with 5'-hydroxylated groups (Chappell et al., 2002). However, this method is restricted to the joining of radioactively labeled compatible and blunt ends only.

4.2. The 2nd decade

The in vitro assay developed by Pastwa et al. (2001) is free of DNA double-strand break type limitations. This strategy is based on incubation of plasmid DNA linearized by the radiomimetic drug bleomycin with human cell extracts and repair products are identified by 1% gel electrophoresis. To make the direct detection approach practical and eliminate the complex and time-consuming Southern blotting or radioactive methods, the fluorescent DNA stain Vista Green was employed. Vista Green was at least 50-fold more sensitive than ethidium bromide. This was the first, novel application of this dye in the detection of NHEJ. Currently, many laboratories are using fluorescent DNA stains similar to Vista Green, like

SYBR Green I and SYBR Gold to name just a few, in DSB repair assays (Diggle et al., 2003; Iliakis et al., 2006; Pastwa et al., 2005; Wang et al., 2003).

In a follow up study, our group has also used DSBs produced by IR and ^{125}I (Pastwa et al., 2003). ^{125}I produces non-ligatable DSB ends with varying overhang lengths, and base loss or modifications proximal to the break site. ^{125}I -labeled triplex-forming oligonucleotides induced DSBs in a target duplex DNA molecule at an efficiency of nearly one DSB/decay (Panyutin and Neumann, 1994). The main conclusion from these data is that as the structural complexity of the DSBs increases, repair efficiency *via* NHEJ becomes limited and eventually inhibited. Also, it was demonstrated for the first time that the DSBs ends produced by ^{125}I possessed 3'-blocking groups. A recent study has confirmed the observation, that 3'-ends are blocked and are in part terminated by phosphate, but an assay involving a ^{32}P postlabeling failed to detect 3'-phosphoglycolate at ^{125}I -induced DSB (Datta et al., 2007). Even though the 3'-end structure has not been reported, Panyutin and Neumann's group have established the 5'-phosphate structure (Panyutin and Neumann, 1994, 1996).

Pfeiffer with coworkers also reported lower efficiency of NHEJ repair with ^{125}I -induced double-strand breaks versus DNA cut with RE (Odersky et al., 2002). In this report a gel assay with the Southern blotting technique was used to measure repair efficiency, and a bacterial transformation/mutation assay with DNA sequence analysis of repair products was used to measure repair fidelity. Both findings have a great impact on understanding of the repair of DSBs caused by ^{125}I and other Auger electron-emitting radioisotopes used in anti-gene radiotherapy (Winters et al., 2003).

As mentioned earlier, many current in vitro DSBs end joining assays have some limitations with regard to substrate modification. Recently, an assay using linear duplex oligonucleotides has been described by the Winters laboratory (Datta et al., 2006). An assay based on hairpin oligos already exists (Beyert et al., 1994), but it requires complex multistep purification and processing steps for incorporation of modifications. Datta et al. (2006) describe an in vitro end joining assay that is simple, quick and similar to the plasmid-based assay that was developed in the same laboratory previously (Pastwa et al., 2001). They demonstrated that 75-bp duplex oligonucleotide with a 5'-end labeled strand is a good substrate for end joining, and that this substrate can be used to introduce DNA structural modifications in the form of defined synthetic lesions and assess their impact upon the DSB end joining reaction. Moreover, it was confirmed that this substrate required DNA ligase IV and Ku to be ligated by a human cell extract. These findings are in agreement with observations of McElhinny and coworkers (Nick McElhinny et al., 2000) and indicate involvement of NHEJ pathway in end joining.

An interesting modification of the Baumann's and West's in vitro NHEJ assay method (Baumann and West, 1998) came from the study of Diggle et al. (2003). In this case extracts from much smaller cell numbers than the original assay were used. For NHEJ product identification, a quantitative non-radiolabelled method using fluorescent dye similar to a previously developed assay (Pastwa et al., 2001) has been used. The main advantage of Diggle et al.'s method is that for the first time an in vitro NHEJ assay can be applied to clinical specimens, and their end joining activity can be assessed within 24 h of receiving samples. Until this time the direct and immediate use of clinical material to make cell extracts and assess NHEJ activity had not previously been investigated.

Biochemical reconstitution is one of the major ways to understand functions of proteins involved in NHEJ pathway. In Lieber's laboratory an in vitro NHEJ assay was developed to support the reconstitution, for the first time, of end joining with DSBs possessing incompatible ends and the purified NHEJ proteins: Ku, DNA-PK $_{cs}$, Artemis, DNA ligase IV/XRCC4, DNA polymerase μ and polymerase λ and terminal deoxynucleotidyl transferase (TdT) (Ma et al.,

2004). For the NHEJ substrate, they used oligonucleotides because in this case the overhang sequence can be changed easily. The substrate was 5'-phosphorylated with unlabeled ATP on both sides and incubated with proteins. Then a PCR assay was used to amplify the joined products with the primers 5' end that were radioactively labeled (Ma and Lieber, 2006). To visualize the PCR products, DNA was resolved by denaturing polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. Subsequently, the sequences of the PCR products were determined. A PCR assay has many advantages when using incompatible DNA ends since end joining is inefficient and difficult to detect. Reconstitution of NHEJ with incompatible DSB ends and the purified core NHEJ proteins is a key approach that has been lacking in the biochemical studies of this pathway.

A different kind of NHEJ reconstitution study was undertaken by Nick McElhinny et al. (2005). The goal of the study was to investigate the role of polymerase μ and polymerase λ in the repair of DSBs by NHEJ. End joining substrates were generated by PCR amplification in the presence of ^{33}P . Repair products were resolved by native PAGE and detected by autoradiography. They showed that polymerase μ and polymerase λ , but not polymerase β (data consistent with Ma et al. (2004)), were similarly recruited by NHEJ proteins to fill gaps when ends have partially complementary overhangs. These findings suggest equivalent roles promoting joining. However, compared to previous studies by Ma et al., this reconstitution system did not include nuclease activity.

To measure the joining efficiency of various incompatible ends, Budman and Chu (2005, 2006) developed a quantitative PCR assay. They reproduced the NHEJ of compatible ends previously reported by Baumann and West (1998) and observed that their cell extract joined the DNA into dimers, trimers, but not into circular monomers. They concluded that absence of circular monomers in their NHEJ reaction was due to binding of the DNA by proteins in the extract. Since they wanted to investigate processing of ends prior to joining, it was critical to prepare DNA substrates free of contaminating DNA fragments. Plasmid DNA was the template for PCR amplification of DNA with various RE sites at the ends. Direct digestion of the PCR products could produce contamination from uncut DNA. Therefore, PCR products were subcloned into a plasmid vector, cleaved with the appropriate RE, gel-purified, and stained with ethidium bromide. Additionally, sequencing of junctions showed an absence of contaminating DNA fragments. Joining efficiency was measured by quantitative PCR. The percentage of DNA ends joined was determined from parallel PCR amplifications of pre-formed DNA. This system was dependent on Ku, DNA-PK $_{cs}$, and XRCC4/ligase IV, the key core proteins of NHEJ in vivo. Processing of incompatible ends prior to joining involved polymerase and nuclease activity and was highly efficient compared to compatible ends.

In a follow up study, in Chu's laboratory, a two-stage assay to separate the processing and ligation steps has been investigated (Budman et al., 2007). The standard assay protocol is similar to that developed previously (Budman and Chu, 2005). Using this assay, Budman et al. wanted to answer the question of whether DNA-PK $_{cs}$ kinase activity or XRCC4/ligase IV are involved in processing or ligation. Briefly, in stage 1 they incubated the DNA substrates with four extract preparations including extract treated with wortmannin (inhibition of DNA-PK $_{cs}$) and extract immunodepleted for XRCC4/ligase IV. For stage 2 they treated half of their purified DNA from the joining reaction with T4 DNA ligase (it has ability to join compatible ends) and left the other half of DNA untreated. Then they measured the end joining efficiency by quantitative PCR and examined the processed ends by PCR amplification and sequencing of the junctions. The assay demonstrated that kinase activity is required for both processing and ligation of DNA ends. In addition, they concluded that XRCC4/ligase IV is required, apart from ligation, for processing of DNA ends. These findings suggest a mechanism for how processing is controlled in vivo during NHEJ.

5. Future prospects of NHEJ assays

It is obvious that in spite of the significant progress that has been made, NHEJ assays are still emerging, and likely to become more sophisticated, automated, efficient and sensitive in the next 20 years. Molecular diagnostics and the need to customize radiation treatment will be the major drivers for new and more efficient assays. The fact that NHEJ assays can now be applied to clinical samples (Diggle et al., 2003) is encouraging and a major step forward. These assays, especially newer variants, will find increasing future application in biomedical research, drug discovery and in prognostication, especially in the area of cancer.

Recent publications apparently show that NHEJ assays are already being used to estimate the potential toxic effect of anticancer drugs and IR in therapies against cancer. Because NHEJ pathway is the major repair pathway in cells (Yu et al., 2008) the ability to determine the DSB repair capacity of individual cancer cells, or sensitivity to ionizing radiations will become increasingly important in the clinic. Knowing the repair capacity of cancer cells isolated from a patient's primary tumor and/or determining if cancer predisposing genes are associated with DSB repair proteins may improve the ability of oncologist to identify patients that will benefit from radiation therapy or chemotherapy.

Interestingly, several studies have also shown that DSB repair capacity varies in cells, and the variation in the general population may influence cancer susceptibility (Sakata et al., 2007; Chistiakov et al., 2008). This suggests that NHEJ assays may become the standard tool for accurate estimating of risk at the molecular level and prediction of the susceptibility to cancer development in a population. It could also be used routinely for identification of individuals with lower capacity to repair DSBs because of dysfunctions in a specific NHEJ pathway and hence more likely to develop certain types of cancers.

It is recognized that treatment failure after radiation therapy maybe associated with different capacities of cancer cells to repair DNA DSBs. This is because of the realization that the genetic alterations in genes involved in NHEJ-mediated repair of DSBs apparently influence radiosensitivity and radioresistance. NHEJ assays are therefore likely to become increasingly important in the future by serving as the basis for identifying patients that are indicated or not-indicated for IR. Specifically, the NHEJ assay will be used to estimate the repair capacities of cancer cells in advance of treatment to enable the stratification of patients into radiosensitive or radioresistance categories, as well as selection of radiation dosage prior to radiation therapy.

Recent direct applications of NHEJ assays in cancer research suggest that NHEJ assays are being actively investigated in the clinic, including their use in assessment of double-strand break repair capacity and risk of developing breast cancer risk (Bau et al., 2007), determination of the toxicity and carcinogenic potential of metals like cadmium (Viau et al., 2008) and prediction of tumor recurrence after therapy in head and neck cancer (Pavón et al., 2008).

6. Conclusions

This year (2008) commemorates the 20th anniversary of the development of the first in vitro NHEJ assay (Pfeiffer and Vielmetter, 1988). During this period, many in vitro systems were established and we have considered some of these NHEJ studies in this review. Some earlier developed assays are still in use, but new assays meet the technical challenges of employing radiation damaged DNA and DNA possessing structurally complex DSBs. They can also be applied to clinical material and they are easy to set up and perform.

Biochemical studies on NHEJ have lagged behind its genetic analysis and there has been a need to change this since biochemical studies can answer many questions that cannot be answered at the DNA level. For example, which enzymatic activities participate in the NHEJ

pathway, whether the processing of DNA lesions near DSB ends occurs before or after end joining, and what additional proteins are involved in such processes? To answer some of these questions, in vitro NHEJ assays have been developed which involve more complex DSBs structures.

In vitro reconstitution with purified proteins and defined DSB substrates can be a key approach to study the biochemistry of processing DNA ends for NHEJ. Since it has recently been suggested that XLF could be a third player in the XRCC4/ligase IV complex, it is now a big challenge to study the joining reactions with all known purified proteins including XLF. Characterization of this system may lead to new insights into the pathway of NHEJ.

Identifying proteins involved in NHEJ can be useful for the identification of novel drug targets. DNA-PK_{cs} inhibitors, such as wortmannin or NU7026, sensitize cancer cells to drugs and radiation (Pastwa and Malinowski, 2007; Yu et al., 2008; Kim et al., 2007). Novel poly (ADP-ribose) polymerase-1 (PARP-1; involved in an alternative NHEJ pathway independent of DNA-PK) inhibitors also act as chemo- and radiosensitizers in vitro and in vivo (Albert et al., 2007; Hay et al., 2005; O'Connor et al., 2007). Taken together, NHEJ assays can become an excellent tool for identifying specific inhibitors of the DNA double-strand break repair reaction and thus could be employed in radiation therapy and chemotherapy in cancer.

The next 20 years will witness newer and more sensitive assays and a wider application of NHEJ assays in clinical diagnosis, prognostication and customization of therapy. This is because of the realization that genetic differences can (a) influence the capacity to repair DNA DSBs, (b) predict susceptibility to cancer/likelihood of cancer recurrence and (c) response to IR. We predict that NHEJ assays will become more sophisticated and ultimately become one of the standard tests for selection of cancer patients for targeted therapy.

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Abbreviations

AFIGE	asymmetric field inversion gel electrophoresis
APE1	apurinic/aprimidinic endonuclease
DNA-PK _{cs}	DNA-dependent protein kinase catalytic subunit
DSBs	DNA double-strand breaks
IR	ionizing radiation
NHEJ	non-homologous DNA end joining
PNK	polynucleotide kinase
PAGE	polyacrylamide gel electrophoresis
PARP-1	poly(ADP-ribose)polymerase
PG	phosphoglycolate
RE	restriction enzyme
TDP1	tyrosyl-DNA phosphodiesterase
XLF	XRCC4-like factor

XRCC4

X-ray cross complementation 4

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Fig. 1.

A simple scheme of non-homologous DNA end joining. Ku binds to the oxidatively induced DSBs. Artemis–DNA-PK_{cs}, removes several nucleotides from single-stranded overhangs, especially when a region of microhomology occurs (transversal lines). DNA polymerases μ and/or λ fill in the gaps of several nucleotides (thin lines). The ligase complex ligates the DSBs.

Fig. 2.

The typical workflow for in vitro non-homologous DNA end joining analysis. (1) Plasmid DNA is subjected to damage by an agent of choice and (2) full length linear DNA containing a single DSB is isolated. (3) Linear substrate DNA is then incubated with cell extract as a source of DNA repair enzymes. (4) Reaction products are identified by agarose gel electrophoresis as unreacted linear substrate (L), or end-joined dimers (D), trimers (T), and other high molecular weight products (HM). Detection methods for repair products vary from ethidium bromide, Southern blotting, autoradiography, PCR to very high sensitivity fluorescent dyes other than ethidium bromide.

Table 1

In vitro non-homologous DNA end joining systems: chronological summary of methodology.

Authors	Source	DNA substrates	Methods of detection	Year	Comments
Pfeiffer and Vielmetter	Xenopus egg	RE-cut plasmid DNA	Southern blotting	1988	The first in vitro NHEJ system
North et al.	Human cell	RE-cut plasmid DNA	Southern blotting	1990	The first mammalian in vitro NHEJ system
Fairman et al.	Human cell	RE-cut plasmid DNA	Ethidium bromide	1992	The first use of ethidium bromide
Beyert et al.	Xenopus egg	Oligonucleotides	Autoradiography	1994	The first use of oligonucleotides substrates
Gu et al.	Xenopus egg	Bleomycin-cut oligonucleotides	Autoradiography	1996	The first use of bleomycin-cut oligonucleotides
Cheong et al.	Human cell	IR-cut genomic DNA	AFGE	1998	The first use of IR-cut genomic DNA
Baumann and West	Human cell	RE-cut plasmid DNA	Autoradiography	1998	The first demonstration of core proteins involvement in NHEJ
Pastwa et al.	Human cell	Bleomycin-cut plasmid DNA	Vistra Green	2001	The first use of Vistra Green
Diggle et al.	Human tissues	RE-cut plasmid DNA	SYBRGreen I	2003	The first use of human tissues
Ma et al.	Purified proteins	Oligonucleotides	PCR/autoradiography	2004	The first NHEJ reconstitution with purified proteins
Budman and Chu	Human cell	RE-cut plasmid DNA	Quantitative PCR	2005	The first use of quantitative PCR

Abbreviations: AFGE, asymmetric field inversion gel electrophoresis; IR, ionizing radiation; RE, restriction enzymes.

Note: Bold font, the new aspects of the analysis.