surgery for differentiated thyroid cancer. Although rhTSH was first approved for clinical use in the United States, most European and certain Asian and South American countries approved rhTSH for ablation of postsurgical thyroid remnants before the FDA action. In this article we review the timelines for rhTSH approval and present the views of experts from around the world regarding the expanding indications for rhTSH in thyroid cancer.* The commentary concludes with an editorial summary.

Timelines for rhTSH Approval†

On November 30, 1998, the FDA issued its first approval of rhTSH, as "an adjunctive diagnostic tool for serum thyroglobulin (Tg) testing with or without radioiodine imaging in the follow-up of patients with thyroid cancer" (1). In the original approval, provisions were made for thyroid cancer patients who were unable to mount an endogenous TSH response to hypothyroidism, such as those with pituitary failure. On September 3, 2000, rhTSH was approved in European Union countries‡ for preparation of Tg testing with or without radioiodine imaging for detection of thyroid remnants and well-differentiated thyroid cancer in adult postthyroidectomy patients maintained on hormone suppression therapy. Low-risk patients with well-differentiated thyroid carcinoma who have undetectable serum Tg levels on thyroid hormone suppression could be followed by assaying rhTSH-stimulated serum Tg levels. These indications were adopted in a similar time period by Liechtenstein, Norway, and Iceland, and similar indications were approved by Ukraine on May 17, 2004.

On February 23, 2005, rhTSH was approved in Europe for pretherapeutic stimulation and low-risk postthyroidectomy adult patients maintained on hormone suppression therapy for the ablation of thyroid remnant tissue in combination with 3.7 GBq (100 mCi) radioactive iodine (RAI). In Australia (2006), Malaysia (2007), Thailand (2007), and Singapore (2007), government agencies approved indications for rhTSH similar to that adapted by the FDA in 2007. As noted by Dr. Yamashita, rhTSH has not been approved for clinical use in Japan. Prior to the approval of rhTSH for remnant ablation in 2007, the FDA approved label changes on March 11, 2004, and January 1, 1006. These dealt with Thyrogen® Tg testing alone and in combination with radioiodine imaging in patients with metastatic disease and a Quality of Life statement, respectively. In 2006 rhTSH was approved for clinical use by many countries in South America (see below).

EXPERT COMMENTARY

Asia and Australia

Shunichi Yamashita—The recent approval of rhTSH as Thyrogen® (Genzyme Corporation, Cambridge, MA) by the U.S. FDA will have a valuable impact in the management of patients with well-differentiated thyroid cancer throughout the world. Until recently, management guidelines in the United States and Europe, as proposed by both the American Thyroid Association Guideline Taskforce (2) and the European Thyroid Cancer Taskforce (3), merely recommended Thyrogen® instead of thyroid hormone withdrawal for the detecting recurrence in patients with thyroid cancer. Now the FDA has expanded the use of Thyrogen® to preparation of patients for ablation of thyroid remnants by RAI. This was based on pivotal clinical studies. These studies suggested that the efficacy of Thyrogen® was similar

*"rhTSH" is used to refer to Thyrogen®, but TSH is a heterogeneous substance. It is likely that Thyrogen® is not completely identical to the multiple molecular forms of human TSH that occur *in vivo*. In the future other TSH-like or TSH antagonist agents synthesized by recombinant technology may become available for clinical use, and the term rhTSH will lose its specificity for Thyrogen®.

†http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.Label_ApprovalHistory#apphist, Approval History, Letters, Reviews & Related Documents, accessed 4/2/2008.

‡Austria, Belgium, Bulgaria, Czech Republic, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Portugal, Romania, Slovak Republic, Slovenia, Spain, Sweden, and United Kingdom.

to thyroid hormone withdrawal in achieving ablation of thyroid remnants but had significantly less side effects than thyroid hormone withdrawal because patients did not need to discontinue their thyroid hormone replacement therapy.

In Japan, however, the official approval to use Thyrogen® even as a diagnostic means has not been issued yet, so that in this area it puts the country behind the United States and Europe. There is no gold-standard how to treat and follow patients with well-differentiated thyroid cancer, which generally has a good prognosis. It is essential to use rhTSH in accordance with standardized guidelines as this is likely to contribute to the care of patients where it is used, and provide experience and evidence to enhance patient care in the future. It is also important to overcome shortages of nuclear medicine facilities and have a better understanding of the risks and benefits of RAI therapy. In this sense more experience with Thyrogen® may be advantageous. Arguing for the approval of Thyrogen® is that it is one of the most effective approaches to facilitate the detection and staging of thyroid cancer and now appears to be an important tool in using RAI to ablate or destroy the remaining thyroid tissue in patients who have had their cancerous thyroids removed. It is my hope that the current FDA decision will promote clinical application of Thyrogen®, both for follow-up diagnosis and to facilitate RAI therapy in Japan and other Asia-Oceania countries where Thyrogen® has not yet been received into medical practice.

Bruce G. Robinson—The use of rhTSH in the management of thyroid cancer has been one of the most significant therapeutic advances in thyroidology in the last decade. In many countries rhTSH is available for diagnostic use with the measurement of Tg and thyroid scanning in the follow-up of patients with differentiated thyroid cancer. The recent FDA approval of rhTSH for immediate postoperative ablation of remnant thyroid tissue will save patients from the need to be made hypothyroid in this period with its attendant morbidity and with arguable cost benefit. The FDA approval follows similar approvals in Australia (2006), Malaysia (2007), Thailand (2007), and Singapore (2007). Of course these approvals are not necessarily associated with patient access since funding for rhTSH is often restricted by the government and out of the reach of many deserving patients. In Australia for instance, the use of rhTSH for diagnostic purposes is restricted to patients who have had psychiatric problems or cardiac dysfunction with thyroid hormone withdrawal, and cost prevents many patients who do not meet these criteria from accessing the drug.

There seems little doubt that rhTSH is at least as effective as withdrawal of thyroxine (T4) in preparing for ablation of thyroid remnants and the morbidity is significantly lower. The FDA approval for this indication brings it into line with most developed countries. It is frustrating that an agent with such clear efficacy is not more widely available for all patients with thyroid cancer for diagnostic and therapeutic purposes. Provided that it is used by clinicians aware of the potential adverse effects in patients with cerebral or spinal metastases, it should be the definitive way to prepare patients for follow-up scans or treatment.

Europe

Christoph Reiners and Markus Luster—In patients with epithelial thyroid cancer, measurement of serum Tg for diagnostic purposes and administration of RAI for imaging and treatment usually require that serum TSH levels be elevated to at least 30 mU/L. As an alternate to thyroid hormone withdrawal, rhTSH can be administered to achieve serum TSH concentrations in the range of 100–150 mU/L 3–24 hours later (4). Approximately 1 day after they peak, serum TSH concentrations are usually still greater than 30 mU/L.** Since its

**The mean apparent elimination half-life of rhTSH is 25±10 hours.

introduction, the use of rhTSH has spread. It is estimated that thousands of doses of rhTSH have been administered to patients in the 7–8 years after its initial approval.

When considering the role of rhTSH as opposed to thyroid hormone withdrawal, quality of life is increasingly recognized as being of great importance. These patients usually have excellent survival but should adhere to a long-term monitoring program. An important difference between RAI imaging after thyroid hormone withdrawal and that after rhTSH administration is that patients are hypothyroid in the former state and euthyroid in the latter. In the euthyroid state there is a substantially higher renal clearance of radioiodine. Therefore, in a recent randomized trial (5) of rhTSH versus thyroid hormone withdrawal to ablate thyroid remnants with 3.7 GBq (100 mCi) ^{131}-I RAI, there was a significantly lower radiation dose to the blood with the rhTSH method (0.11±0.028 vs. 0.17±0.061 mGy/MBq, $p<0.0001$). A trade-off for the lower radiation exposure to extrathyroid tissues could well be a lower pool of circulating radioiodine available for targeting healthy or cancerous thyroid tissue, resulting in lower re-uptake in such tissue.

Numerous controlled studies in diagnostic and ablation settings have demonstrated rhTSH-aided scanning to have an equivalent and high sensitivity compared to thyroid hormone withdrawal. rhTSH-facilitated ablation of thyroid remnants also provides comparable results to ablation in the hypothyroid state. An advantage of rhTSH is that it allows more predictable timing and, often, more convenient scheduling of imaging or ablation procedures.

Although not approved as an indication, much experience has been accumulated using rhTSH as an adjunct to treatment of distant metastatic disease. No prospective, randomized trial of rhTSH-aided RAI treatment of thyroid cancer metastasis has been conducted, however. Assessment of the results for rhTSH for this purpose is complicated by the late- or end-stage status of most patients who have been treated to date and to the heterogeneity in patient characteristics and severity of underlying disease. A considerable drawback to using rhTSH is its high cost. Studies have suggested, however, that society and patients are repaid by improvement in productivity, fewer missed workdays, and maintenance of an acceptable quality of life.

Martin Schlumberger—We use rhTSH in low-risk thyroid cancer patients for radioiodine ablation of normal thyroid remnants after surgery, and for assessing cure during follow-up (2,3,6). The rationale for using rhTSH is based on prospective studies that demonstrated a similar efficacy of rhTSH and withdrawal, and that were performed either in patients following rhTSH and then following withdrawal (7) during follow-up or in randomized patients either to withdrawal or rhTSH administration for radioiodine ablation (8). The results of these studies were confirmed by many uncontrolled and mostly retrospective studies (6). In these patients, the advantages of using rhTSH instead of withdrawal are that rhTSH avoids hypothyroidism (and potential associated morbidity) and improves the quality of life (9); the use of rhTSH also reduces the societal cost of the procedure (10-12); finally, in case of administration of radioiodine, the renal clearance of radioiodine is faster, resulting for a similar administered activity in a lower dose by at least one third to extrathyroid tissues, and permitting a shorter stay in the radiation protection ward (6). Therefore, in our clinical practice, whenever stimulation by TSH should be performed in low-risk patients, we routinely use rhTSH instead of withdrawal.

There are still several questions concerning its use. The recommended activity to be administered for ablation of normal thyroid remnants was 3.7 GBq (100 mCi), but several non-randomized studies using 1.11 GBq (30 mCi) and a randomized study (13) comparing 1.85 and 3.7 GBq (50 and 100 mCi, respectively) have shown promising results. Prospective randomized studies on large numbers of patients are being performed comparing in terms of

efficacy and of medicoeconomic evaluation the stimulation method (either withdrawal or rhTSH) and the activity administered (30 versus 100 mCi). This should permit the definition of the optimal procedure.

The discovery of many thyroid cancers at an early stage and the improvement of initial treatment procedures have permitted a decrease in the rate of persistent or recurrent disease. It is important to perform restaging at around 9–12 months after initial treatment to assess if the patient is at very low risk of recurrence (high probability of cure). In low-risk patients who have previously undergone thyroidectomy and radioiodine ablation, restaging is achieved using rhTSH stimulation and neck ultrasound. If these are negative, follow-up consists of Tg determination on thyroxine and repeat ultrasound at regular intervals, and the daily dose of thyroxine can be reduced. In these patients, there is no need to repeat rhTSH stimulation test in the absence of suspicious abnormalities (14). Patients with low positive Tg at 9–12 months should undergo repeated rhTSH testing, while those with Tg above the institutional cut-off are treated. The newer "supersensitive" Tg assays may have a role in this setting, since it permits the discovery at an early stage of a higher percentage of persistent or recurrent disease during thyroxine treatment, but at the expense of a larger number of low but detectable serum Tg levels (15). Up to now, rhTSH stimulation provides the most reliable assessment of cure, and further studies are needed to assess the significance of these very low Tg levels.

In patients with known tumoral foci, the few available studies have shown that the radiation dose delivered to neoplastic foci following rhTSH is probably far lower than that delivered by a similar activity following withdrawal (16). Up to now, this is the rationale to restrict the routine use of rhTSH to low-risk patients. However, larger doses of RAI may be administered in these patients, and this is permitted by the higher renal clearance of radioiodine. A prospective study comparing dosimetry following rhTSH and then withdrawal is needed, before using rhTSH as a routine preparation for radioiodine treatment of neoplastic foci.

In conclusion, the availability of rhTSH has been a major progress in the management of thyroid cancer patients. rhTSH should be used routinely in low-risk patients whenever stimulation by TSH is required.

North America

David S. Cooper—Although studies published in 2001–2003 strongly suggested that Thyrogen® could be used for remnant ablation, it was not until the results of the international randomized controlled trial (8) became known that I began to use Thyrogen® routinely for this purpose in low-risk patients. And since it was approved for this indication in Europe in 2005, it was only a matter of time before the FDA would approve it for this indication here in the United States. The advantages of using Thyrogen® for remnant ablation include simplicity, the possibility of lower body radiation exposure (5), and maintenance of quality of life (9). The disadvantage is the cost, although some would argue that Thyrogen® is cost effective from a pharmacoeconomic perspective (12). In my view, the admonition in the package insert that states "Due to the relatively small clinical experience with Thyrogen in remnant ablation, it is not possible to conclude whether long-term thyroid cancer outcomes would be equivalent after use of Thyrogen® or use of thyroid hormone withholding for TSH elevation prior to remnant ablation" is unduly conservative, since there is no logical reason that long-term outcomes would be any different with the two methods of remnant ablation.

For me, the major unanswered question is whether Thyrogen-mediated ablation can be performed with radio-iodine doses less than 3.7 GBq (100 mCi). Limited data suggest that this is the case (13), with 1.85 GBq (50 mCi) and 3.7 GBq (100 mCi) being equivalent in a small number of patients. On the other hand, ablation rates are lower with Thyrogen® using 1.11 GBq (30 mCi) (17). Therefore, whether the lower total body radiation dose observed with

Thyrogen® compensates for the need to use a larger dose of RAI than one might ordinarily use in some patients (i.e., 30 mCi) remains an important unanswered clinical question. Another issue for further study is whether Thyrogen® can be used for remnant ablation in high-risk patients.

Finally, the complexities of performing a diagnostic ^{123}I pretreatment scan when using Thyrogen® are daunting, since the scan needs to be inserted before the therapy dose in a very tight time window. Granted, many experts do not request pretreatment scans for their patients (2), but for those such as myself who have routinely done so for a variety of reasons, we have abandoned these in most cases because of the logistic difficulties, and simply administer a treatment dose. Another option, which would require Thyrogen® to be used on 2 successive weeks (1 week for diagnostic scanning and 1 week for treatment), is financially untenable for most patients. Thus, the approval of Thyrogen® will, almost certainly, lead to the demise of routine pretherapy scans.

I welcome the recent FDA approval of Thyrogen® for remnant ablation, and hope that future research will solve some of the important clinical questions that remain.

Ernest L. Mazzaferri—The introduction of recombinant human TSH-α (rhTSH) had an almost immediate impact on the management of differentiated thyroid carcinoma. The first two prospective clinical studies (7,18) of rhTSH demonstrated that administration of the drug was a safe and effective means of stimulating thyroidal radioiodine uptake and the release of Tg, while maintaining the patient in a euthyroid state and protecting the patient's quality of life (9). Patients shortly began asking their physicians for rhTSH-stimulated diagnostic whole-body ^{131}I scanning rather than undergoing thyroid hormone withdrawal. The main impetus for this was that diagnostic whole-body ^{131}I scanning had been for many years the principal means of identifying residual differentiated thyroid cancer. As a consequence, patients typically underwent yearly thyroid hormone withdrawals over an extended period, leaving them to endure recurrent symptoms of hypothyroidism. I vividly recall speaking to a large group of patients with thyroid cancer at the national Thyroid Cancer Survivors Association who not only expressed keen interest in rhTSH, but spoke disparagingly of their various bad experiences with thyroid hormone withdrawal. They knew without reading the medical literature that short-term hypothyroidism after levothyroxine withdrawal is associated with a significant decline in quality of life, and that hypothyroid symptoms were abolished by the continued administration of levothyroxine during rhTSH stimulation. Indeed, patients often became aware of this before their doctors recognized the major clinical impact that the drug would have on their practice.

By 1985 it had become apparent that serum Tg measurements performed after thyroid hormone withdrawal were more accurate in identifying residual tumor than Tg measured during levothyroxine suppression of TSH (19). The second prospective rhTSH study (7) found that rhTSH-stimulated serum Tg measurement was as accurate in identifying residual tumor as Tg measurements made during thyroid hormone withdrawal. A number of clinical studies subsequently found this to hold true in day-to-day practice (20-23). As a result, TSH-stimulated serum Tg measurement has become a key step in identifying patients with persistent tumor, which has been integrated into the American and European Thyroid Association guidelines for the management of differentiated thyroid carcinoma (2,3).

The recent approval of rhTSH preparation for ^{131}I thyroid remnant ablation is most welcomed by patients, many of whom opted for this choice well before the drug had been approved for this indication by the FDA. From my stand-point, the most important finding of the initial international prospective randomized study by Pacini *et al.* (8), which compared thyroid hormone withdrawal with rhTSH for remnant ablation, was that rhTSH-stimulated ^{131}I ablation was associated with 33% less total body radiation than thyroid hormone withdrawal. Probably

the two most worrisome problems about ^{131}I remnant ablation for patients and physicians alike are the adverse effects of the radioisotope on the oral cavity, parotid glands, and lachrymal ducts, and the possibility of developing radiation-induced secondary cancers. Both sets of complications are related to the cumulative radiation delivered by ^{131}I (24). Thus, anything that lowers total body irradiation is a welcome finding for patients, particularly in light of two recent studies (25,26) that found empirically administering ^{131}I in lieu of dosimetry is prone to underestimating total body radiation exposure. Even better news is the randomized prospective study by Pilli *et al.* (13) that found therapeutic ^{131}I activities of 1.85 GBq (50 mCi) and 3.7 GBq (100 mCi) are equally effective for thyroid remnant ablation in patients prepared with rhTSH, even in the presence of lymph node metastases.

The introduction of rhTSH into clinical practice has sparked research that no longer centers around the indication for total thyroidectomy in the management of thyroid cancer, something that was the main issue debated over decades. Now our debates center upon follow-up strategies and the role of more sensitive Tg assays in the follow-up management. All of this resulted from the introduction of a new drug, rhTSH, in a field that has had a paucity of new therapeutic drugs introduced over the past several decades.

Richard J. Robbins—Recombinant human TSH (rhTSH; Thyrogen®) was developed in the late 1980s as collaboration between academic physician-scientists and a young biotechnology company, Genzyme, Inc. It was the first recombinant human hormone to have a direct impact on the management of patients with thyroid disease. rhTSH was initially approved as an aid in surveillance testing for survivors of thyroid cancer. The FDA approval of rhTSH in November 1998 was based on two phase III trials, which demonstrated that the diagnostic accuracy of radioiodine and serum Tg testing was similar whether patients were prepared by thyroid hormone withdrawal or by rhTSH (7). Although the sensitivity of the whole-body radioiodine scans was somewhat lower following rhTSH preparation, the diagnostic accuracy of the rhTSH-stimulated serum Tg level was equal or better than preparation by hormone withdrawal.

While rhTSH was first developed as a diagnostic agent, it was clear to many thyroidologists that this hormone might also serve as an alternate means of increasing sodium-iodide symporter activity to aid in radioiodine remnant ablation. Perros (27) was the first to report that rhTSH could be used in the remnant ablation setting, while patients continued on thyroid hormone. The first series of successful rhTSH-assisted remnant ablation was reported in 2001 from the Memorial Sloan-Kettering Cancer Center (28). Many short retrospective reports have subsequently confirmed these findings. With the support of the Genzyme Corporation, a group of clinical investigators established and conducted an international randomized controlled trial comparing remnant ablation rates between patients prepared by thyroid hormone withdrawal or by rhTSH (8). In a study of 63 thyroid cancer patients, this team found that radioiodine remnant ablation rates, after administering 3.7 GBq (100 mCi), was comparable between the groups. Those who were prepared by rhTSH had a better quality of life and a longer effective half-time of radioiodine in the remnant, and they received less radiation to the blood (5,8). A subsequent report by Italian investigators (13) found similar high remnant ablation rates at 8 months using only 1.85 GBq (50 mCi). Nevertheless, until further studies determine the most effective amount of ^{131}I to be administered, 3.7 GBq (100 mCi) should be the standard.

The use of rhTSH to prepare patients for radioiodine remnant ablation was approved by the European Medicines Agency in 2005. The U.S. FDA requested a longer term clinical follow-up study to confirm the short-term imaging-based conclusion. According to the new FDA package insert, a 3.7-year follow-up study of patients from the randomized controlled study (8) found that 100% of evaluable patients in both groups had no visible uptake in the thyroid bed or total uptake of < 0.1%. No patient in either group had a definitive cancer recurrence.

Based on this finding, in December 2007, the FDA approved the use of rhTSH in the United States as an adjunctive treatment for radioiodine ablation of thyroid remnants in patients who have had total or near-total thyroidectomy for well-differentiated thyroid carcinoma without evidence of metastatic disease.

Unfortunately, the issue of the term "metastatic disease" has not been fully clarified in the new package insert. There is a very high incidence of loco-regional nodal metastases in patients with papillary thyroid carcinoma. These loco-regional nodal metastases are not considered distant metastases, and therefore rhTSH ablation could be utilized in those who have N1 disease. However, it seems clear, at present, that the FDA does not intend that Thyrogen® be used routinely to treat distant metastases. Unfortunately, it is very unlikely that any federal or industrial organization will support the cost of a randomized controlled trial of rhTSH-assisted radioiodine therapy of metastatic differentiated thyroid carcinoma. This is unfortunate, as many patients with metastatic disease postpone or avoid therapy with radioiodine because of the reluctance to become hypothyroid.

The FDA Indications and Usage statement includes this indication. It reads, "Thyrogen® treatment and testing may be used in patients who are either unable to mount an adequate endogenous TSH response to thyroid hormone withdrawal or in whom withdrawal is medically contra-indicated." Although there are no prospective trials that have tested the safety or efficacy of rhTSH-assisted radioiodine treatment of metastatic disease, retrospective reports of compassionate use cohorts do exist. In aggregate, they suggest that those who cannot make TSH or who cannot medically tolerate hypothyroidism may benefit from rhTSH-assisted high-dose radioiodine therapy for distant metastases (29,30). The risks include expansion (sometimes acutely) of metastatic lesions, and hyperthyroidism in those with a significant thyroid remnant or T4-producing metastatic lesions. After many years of using both approaches, I am convinced that rhTSH-assisted radioiodine therapy of metastases, which takes up iodine, has been as successful as the same therapy in patients following thyroid hormone withdrawal. When considering high-dose radioiodine therapy for metastases, the use of blood and whole-body dosimetry should be strongly considered in order to avoid over- or under-treatment, especially in the very young or old (25).

Many times during my career, I have had thyroid cancer patients referred from other hospitals months after having had a thyroidectomy. They were told to stay off thyroid hormone replacement until they saw an endocrinologist. Some patients have arrived in an advanced state of hypothyroidism because their follow-up was not arranged in a timely manner. With this expanded indication for rhTSH, it may become routine for patients to be placed on thyroid hormone replacement at the time of surgery, and then undergo remnant ablation, if indicated, without ever having to be hypothyroid. The cost-benefit analysis and the improved quality of life that these patients gain by not becoming hypothyroid appears to outweigh the expense of the Thyrogen® medication (6,10).

In summary, thyroidologists should applaud the FDA for expanding the indications for the use of rhTSH, and we should thank the Genzyme Corporation for supporting the long-term studies that were needed to assure the FDA that this approach was safe and effective.

South America

Rui M.B. Maciel and Rosa P.M. Biscolla—Following the European approval for the preparation of patients for thyroid ablation with rhTSH and 3.7 GBq (100 mCi) ^{131}I on levothyroxine (L-T4) in 2005 (European Medicines Agency 2005, Decision C478 of 23=02=2005), agencies in charge of drug regulations of several Latin American countries also have approved the use of rhTSH to prepare patients to destroy thyroid remnants after surgery. Thus, ^{131}I ablation using rhTSH was approved in several countries as Argentina, Brazil, Chile,

Colombia, Mexico, and Peru (Brazilian Medicines Agency (ANVISA), Decision 4034 of 08=12=2006). In Colombia rhTSH was approved to prepare only low-risk patients for thyroid ablation and a minimum of 3.7 GBq (100 mCi) ^{131}I therapeutic activity must be used, but in Brazil, Argentina, and Mexico, however, physicians can use any activity of ^{131}I and the use of rhTSH on L-T4 is not restricted for low-risk patients.

Although in Brazil we do not have any recommendation about which ^{131}I activity is better and which type of patients should be prepared with rhTSH, we believe, at this time, that we should use rhTSH only for low-risk patients on L-T4 (T1-T2, N1 and M0, patient age < 45 years) and to employ a minimum dose of 3.7 GBq (100 mCi) ^{131}I therapeutic activity (31).

Several aspects should be addressed in future studies to delineate the best strategy for the use of rhTSH to facilitate the postsurgical thyroid ablation, since recent reports do not clearly define the best effective activity of ^{131}I, the time and utility for low-iodine diet, the utility of a short L-T4 stoppage, the use of levo-triiodothyronine (L-T3) instead of L-T4, and the appropriate dose of rhTSH (5,13,32).

Geraldo Medeiros-Neto—There is no consensus that thyroid remnant ablation preceded by rhTSH is indicated for low-risk patients after total thyroidectomy. In a recent publication (31) thyroid ablation was recommended for many patients after total thyroidectomy for thyroid cancer but not for those whose risk was low. My informal consultation with several prominent clinical endocrinologists in Brazil, however, indicated that in almost all patients most would recommend an out-patient dose of RAI (1.11 GBq, 30 mCi) preceded by rhTSH. The rationale is to have a better imaging of residual thyroid tissue by whole-body scan during follow-up and, more importantly, to be able to use serum Tg as a marker of active disease. In other countries like Chile, thyroid ablation is favored for low- and high-risk patients with thyroid cancer. rhTSH is seldom used for this purpose, however, as it only became available in 2007 and is still quite expensive. In Argentina, rhTSH preceding ablation with RAI is generally used for patients considered to be at high risk. Low-risk patients are treated only with total thyroidectomy. A recent paper from Brazil (33) recommended a conservative approach with no postsurgical RAI ablation for patients with small, apparently single, thyroid cancers. Patients with thyroid cancer who have been operated on by surgeons without training in thyroid surgery often have large thyroid remnants. These patients, when referred to a University Hospital, are often treated with RAI ablation preceded by rhTSH.

The cost of rhTSH is an important consideration for patients who are candidates for its use in preparing them for RAI ablation. In most countries of Latin America, for the general population, either there is no refund from the National Health Plan or this modality of treatment is only available at Public and University Hospitals. Patients with private health insurance are usually reimbursed up to 70% for the cost of rhTSH. If the health insurance company refuses to reimburse the cost of rhTSH, most courts will decide in favor of the patient. In fact, in Brazil, almost all health insurance companies are paying the total cost because of previous court decisions. Moreover, free assistance regarding the patient's legal rights is now provided by a number of private institutions.

In conclusion, there is a strong tendency in both low- and high-risk patients, for clinical endocrinologists in Latin America to use ablation with 1.11 GBq (30 mCi) or higher doses of RAI in cancer patients after thyroid surgery and to employ Thyrogen® to prepare patients for ablation. The major restriction to this is that patients may not be able to pay for the treatment and lack private insurance. In this case they will need to wait for many months to obtain the treatment in a public or university hospital.

Editorial and Summary‡‡

There is general agreement among the experts featured here that rhTSH is an effective method to prepare patients with low-risk thyroid cancer for postsurgical ablation of thyroid remnants with RAI and that the 2007 decision of the FDA to add this as an indication will benefit patients in the United States. Other areas of unanimity include the impression that rhTSH administration provides better quality of life than thyroid hormone withdrawal by avoiding hypothyroidism. Expense was recognized as a major drawback, particularly in countries where there is little insurance coverage or rhTSH is not covered by the prevalent insurance programs. Counterbalancing this was the belief that the expense of rhTSH is made up for by better productivity than occurs during hypothyroidism when thyroid hormone is stopped.

We agree with Dr. Robbins' observation that some patients who are scheduled for remnant ablation after thyroid hormone withdrawal have an unanticipated delay in their treatment, resulting in severe hypothyroidism. Sometimes this is due to noncompliance on the part of the patient, sometimes to scheduling conflicts, and sometimes to the physician's efforts to reach an acceptable TSH level before administering RAI. The frequency that this occurs is difficult to ascertain as instances of inappropriately delayed RAI treatment are not likely to be reported. Use of rhTSH, however, avoids this problem.

There were few overt disagreements among commentators, but there were gray areas and controversial issues that surfaced. One concerns the ablative dose of radioiodine required for remnant ablation after rhTSH. Some believe we should use the 3.7 GBq (100 mCi) dose used in the randomized study by Pacini *et al.* (8). Others feel that the recent study of Pilli *et al.* (13) supports the use of doses as low as 1.85 GBq (50 mCi), and still others believe that 1.11 GBq (30 mCi) is sufficient.

Although not discussed in some of the commentaries, there is ongoing debate of the utility of pretreatment diagnostic radioiodine scans, usually with ^{123}I, in low-risk patients. Using the standard two-dose rhTSH protocol does not allow for diagnostic whole-body scans and conventional timing of treatment. Some experts are abandoning the diagnostic scan, some avoid rhTSH so they can do both the diagnostic scan and treatment, and others are getting creative by giving rhTSH on day 1 and 2, a ^{123}I dose on the afternoon of day 2, a ^{123}I whole-body scan on the morning of day 3, and ^{131}I treatment on the afternoon of day 3. In this scenario, however, the decision to treat is based on whether or not there are residual postthyroidectomy remnants or for dose adjustment in the presence of distant metastatic disease. As emphasized by several of the commentators, rhTSH is not currently approved by the FDA to prepare patients with metastatic disease for RAI treatment. Nonetheless, it is important to develop protocols for rhTSH-facilitated ablation that incorporates a pretreatment whole-body scan.

We are in general agreement with the opinions of the commentators that rhTSH is definitely valuable in preserving quality of life as opposed to thyroid hormone withdrawal. This is certainly of great personal value to patients and their families. It is difficult to calculate the exact economic impact on the individual and society, although a few studies have examined this effect (12,34). We also agree with the commentaries that whole-body irradiation is reduced by using rhTSH rather than thyroid hormone withdrawal, and this should be a factor in reducing the risk of a second cancer.

One of the most controversial areas, however, concerns which patients need radioiodine remnant ablation. The decision for use of radioiodine for remnant ablation has been based on the impact of this treatment on overall survival, disease-free survival, and ability to monitor patient with sensitive testing (neck ultrasound and sensitive serum Tg testing). Most experts

‡‡Editors Haugen, Emerson, and Pacini.

agree that patients with stage III and VI (AJCC, TNM) disease benefit from radioiodine remnant ablation, which is supported by the evidence (35). Evidence for effectiveness of remnant ablation in low-risk stage I patients, however, is lacking. A therapy-directed approach has been proposed, which divides patients into three basic risk categories when considering radioiodine therapy. These are very low risk, such as a solitary tumor less than 1–1.5 cm; high risk, such as a tumor with extrathyroid extension and/or distant metastatic disease; and low to moderate risk, which is intermediate (35). Patients with very low-risk disease are unlikely to benefit from radioiodine remnant ablation, and many patients with low-to-moderate risk disease may not benefit, especially younger patients with smaller tumors (<2 cm) and no worrisome features (extensive lymph node [LN] involvement and aggressive histology). Future research should focus on who would benefit, based on disease-free survival, from radioiodine remnant ablation.

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Current Topics in DNA Double-Strand Break Repair

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DNA repair/Homologous recombination/Nuclear foci/Non-homologous end joining.

DNA double strand break (DSB) is one of the most critical types of damage which is induced by ionizing radiation. In this review, we summarize current progress in investigations on the function of DSB repair-related proteins. We focused on recent findings in the analysis of the function of proteins such as 53BP1, histone H2AX, Mus81-Eme1, Fanc complex, and UBC13, which are found to be related to homologous recombination repair or to non-homologous end joining. In addition to the function of these proteins in DSB repair, the biological function of nuclear foci formation following DSB induction is discussed.

1. INTRODUCTION: AT the broken DNA ends

Ionizing radiation (IR) induces a variety of DNA lesions, including single- and double-strand breaks, DNA-protein cross-links, and various base damages. A DNA double-strand break (DSB) is one of the most serious threats to cells because it can result in loss or rearrangement of genetic information, leading to cell death or carcinogenesis. There are at least two repair pathways which can repair DSBs: (1) non-homologous end-joining (NHEJ)- and/or micro-homology-mediated recombination, and (2) homologous recombination (HR)-mediated repair.[1)] These damage responding repair pathways are thought to be regulated by several major steps. First, a sensor protein (probably, ATM or Rad50/Mre11/NBS1 complex) recognizes damage induction by radiation. Second, mediator proteins receive a structural modification by the sensor protein(s), and this modification is converted to a compatible form for signal amplification by transducer proteins. These transducers amplify the signal, and finally, effector proteins accomplish enzymatic reactions of DNA end processing, rejoining, or cell cycle regulation. Figure 1 shows a brief overview of relationship among radiation-DSB responding factors. When DSBs are generated, ATM protein kinase is activated and relocates through an interaction with Rad50/Mre11/NBS1 complex.[2)] Then ATM phosphorylates histone H2AX and many other substrate proteins including Artemis, MDC1, NBS1, p53, Chk2, and DNA-PKcs kinase. ATM-phosphorylated proteins activate cell cycle checkpoints, NHEJ repair pathway, and HR repair-related pathways. Hence, ATM kinase, whose mutation causes a genetic disorder, ataxia-telangiectasia (AT), at the broken DNA ends is an central regulator of the DSB responding pathway. In addition to signal transduction, many proteins involved in damage response, including activated ATM itself, form nuclear foci (see chapters 3, 7, and Fig. 7). Recently, it has been found that proteins involved in HR pathway are often ubiquitinated and this seems to be essential for HR repair (chapter 6).

In this review, we summarize current topics in DNA repair with a focus on the function of proteins related to HR repair (chapters 4, 5, and 6), a novel NHEJ pathway that is mediated by 53BP1 (chapter 2), and the biological function of nuclear foci formation of damage sensor or mediator proteins (chapters 3 and 7).

2. 53BP1-dependent repair pathway for X-ray-induced DNA damage

DSBs activate signaling responses, termed cell-cycle checkpoints, which monitor DNA damage and transduce signals to coordinate repair and cell cycle progression.[3)] One

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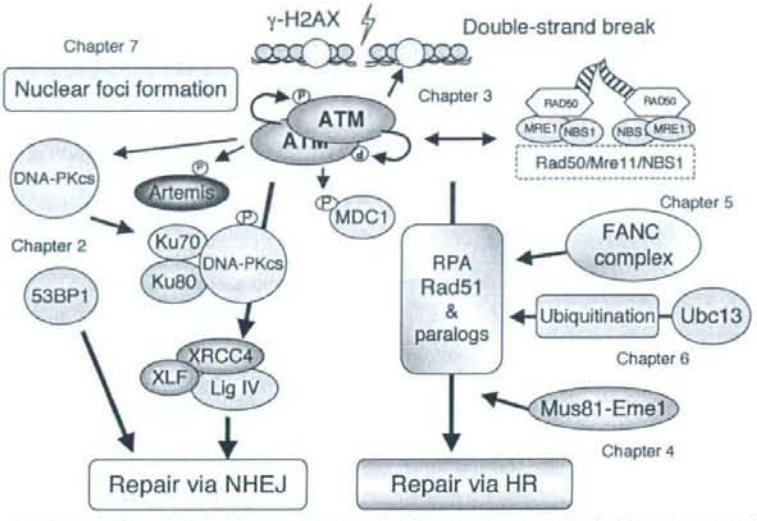


Fig. 1. Proteins related to DNA double strand break repair. Relevant chapter numbers in this review are indicated.

of the key players of cell-cycle checkpoints is the tumor suppressor protein p53. p53 is activated and posttranscriptionally modified in response to DNA damage. These modifications include phosphorylation by ataxia telangiectasia mutated (ATM), a central signaling kinase in the response to DNA damage.[4] p53 transcriptionally activates genes involved in cell cycle control, DNA repair and apoptosis, and participates in the maintenance of the genome integrity after DNA damage.[3]

Using the yeast two-hybrid system, 53BP1 was identified as a protein that binds to wild type p53.[5,6] Human 53BP1 consists of 1972 amino acid residues, the C-terminus of which contains tandem BRCA1 C-terminus (BRCT) motifs. 53BP1 binds to the DNA-binding domain of p53 through 53BP1's BRCT motifs.[7,8] BRCT domain is found in a large number of proteins involved in the cellular responses to DNA damage, suggesting 53BP1's roles in these aspects. Consistently, 53BP1 rapidly forms discrete nuclear foci in response to γ-radiation.[9,10] These foci colocalize with phosphorylated H2AX (γ-H2AX), a marker of DNA DSBs, indicating that 53BP1 relocalizes to sites of DNA DSBs in response to γ-radiation. The minimal domain for focus formation consists of tandem Tudor motifs,[11] which have been reported to associate with various methylated lysine residues in histone H3 and H4. These include lysines K79 in histone H3 and K20 in histone H4.[12,13] Although methylation of histone H3 K79 is unaltered in response to DNA damage, K79 lies in the nucleosome core, and is inaccessible under normal conditions. Because of this, 53BP1 is proposed to sense changes in higher-order chromatin structure.[12]

53BP1 becomes hyperphosphorylated in response to γ-radiation.[10,14,15] ATM-deficient cells show no 53BP1 hyperphosphorylation, and inhibition of phosphatidylinositol 3-kinase family by wortmannin strongly inhibited γ-radiation-induced hyperphosphorylation. In addition, 53BP1 is readily phosphorylated by ATM *in vitro*. These results suggest that 53BP1 is an ATM substrate that is involved in cellular responses to DSBs. However, there is some evidence that 53BP1 have a role in DNA damage signaling upstream of ATM. Analysis of mammalian cell lines depleted in 53BP1 expression through small interfering RNA revealed that 53BP1 is required for accumulation of p53, G2-M checkpoint, intra-S-phase checkpoint, and optimal phosphorylation of at least a subset of ATM substrates such as Chk2, BRCA1 and Smc1 in response to radiation-induced DNA damages.[16,17,18] These results indicate that 53BP1 is a central mediator of the DNA damage checkpoints.[16]

The Tudor motifs also stimulate end-joining by DSB repair proteins DNA ligase IV/Xrcc4, but not by T4 DNA ligase *in vitro*.[19] This suggests that 53BP1 has the potential to participate directly in the repair of DNA DSBs. DSBs are repaired by two major pathways: HR and NHEJ.[20,21] HR primarily uses the undamaged sister chromatid as a DNA template allowing for accurate repair of the lesions, and functions in late S-G2 phase. NHEJ is an error-prone joining of DNA ends with the use of little or no sequence homology, and plays a major role in the repair of IR-induced DSBs, especially during the G1 phase of the cell cycle when sister

chromatids are not available.[22] Riballo and their colleagues proposed a model for the repair of IR-induced DSBs during the G1 phase in mammalian cells, in which the majority of DSBs are rejoined by the "core NHEJ", but repair of a subfraction of DSBs requires Artemis, an endonuclease required for processing the hairpin intermediate generated during V(D)J recombination.[23] The "core NHEJ" is composed of Lig IV/Xrcc4, Ku70/Ku80, and DNA-PKcs. Artemis is a downstream component of ATM-dependent signaling in DSB repair, and the ATM/Artemis-dependent repair pathway also requires proteins locating to sites of DSBs, including 53BP1.[23] However, in chicken DT40 cells, 53BP1 seems to contribute to survival of cells irradiated with IR during the G1 without Ku70 or Artemis. We established 53BP1-deficient chicken DT40 cells.[24] 53BP1-deficient cells show increased sensitivity to X-rays during G1 phase. Although intra-S and G2/M checkpoints are intact, a frequency of isochromatid-type chromosomal aberrations is elevated after irradiation in 53BP1-deficient cells. Furthermore, disappearance of X-ray-induced γ-H2AX foci is prolonged in 53BP1-deficient cells. Thus, the elevated X-ray sensitivity in G1 phase cells is attributable to repair defect for IR-induced DNA-damage. Epistasis analysis revealed that 53BP1 is non-epistatic with Ku70 and Artemis, but epistatic with DNA ligase IV. Strikingly, disruption of the *53BP1* gene together with inhibition of phosphatidylinositol 3-kinase family by wortmannin completely abolishes colony formation by cells irradiated during G1 phase. These results demonstrate that there is a 53BP1-dependent repair pathway which is distinct from the Ku70-dependent and Artemis-dependent NHEJ pathways (Fig. 2).

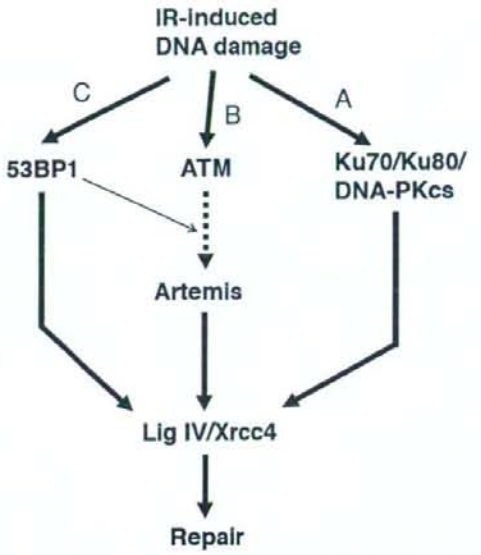


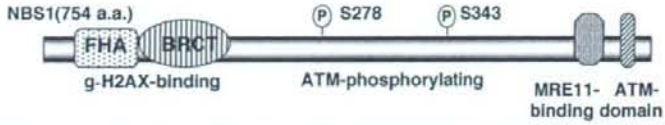
Fig. 2. Model of the repair pathways for IR-induced DNA damage in G1 phase cells. A, B and C represent the core NHEJ, ATM/Artemis-dependent and 53BP1-dependent pathways, respectively. The dotted arrow represents the minor pathway in DT40 cells. The thin arrow represents a possible interaction resulting from the scaffold function of 53BP1.

The 53BP1-dependent pathway made a larger contribution to cell survival in G1 than in early S phase,[24] suggesting that the 53BP1-dependent pathway is regulated at the G1 to S phase transition by mechanisms distinct from the other two pathways. It has been shown that 53BP1-deficient mice have intact V(D)J recombination but impaired class switch recombination.[25,26] It is unclear whether the 53BP1-dependent repair pathway is involved in class switch recombination. However, if, as proposed,[27] class switch recombination occurs in the G1 phase of the cell cycle, it is possible that, in vertebrates, class switch recombination is the main stage at which 53BP1 participates in DNA damage repair.

3. Role of NBS1 and histone H2AX in DNA double-strand break repair

Nijmegen breakage syndrome (NBS) is a radiation-hypersensitive genetic disorder. NBS and AT show the similar cellular phenotypes such as radiation-hypersensitivity, chromosomal instability and radiation-resistant DNA synthesis.[28] So far, it has been clarified that the responsible gene product of NBS, NBS1, interacts with ATM (the responsible gene product of AT syndrome) and this interaction is indispensable for the recruitment of ATM to DSB sites and activation of ATM kinase.[29] Hence, the functional interaction between NBS1 and ATM is important for the regulation of cell cycle checkpoints. Previously, we reported that NBS1 formed a complex with MRE11 nuclease and RAD50 and worked for HR repair in DT-40 chicken cells.[30] Moreover, NBS1 forms the complex with γ-H2AX in response to DSB damage, and this interaction is essential to the recruitment of NBS1 to DSB sites.[31] These facts suggest that the NBS1 complex may function for DSB repair together with ATM and γ-H2AX in human cells. NBS1 has BRCT and FHA domains in the N-terminus, ATM-phosphorylating sites in the central region, and hMRE11 and ATM-binding sites in the C-terminus (Fig. 3). Therefore, we investigated the role of these domains for HR repair using a DR-GFP assay.[32]

The mutation of NBS1 in BRCT, FHA or MRE11-binding domain decreased HR activity, and NBS cells expressing these mutated NBS1 cannot form DSB-induced MRE11 foci. These results indicate that the recruitment of MRE11 to the DSB site by NBS1 is important for HR activity. On the other hand, the mutation in ATM-phosphorylating or ATM-binding sites did not influence the HR activity. Moreover, AT cells showed an HR activity at a similar level as



mutated site	FHA	BRCT	ATM-phosphorylating	MRE11-binding	ATM-binding
HR activity	–	–	+	–	+
NBS1 foci	–	–	+	+	+
MRE11 foci	–	–	+	–	+

Fig. 3. Characteristic domains of NBS1. The domains (FHA, BRCT, MRE11-binding), which are essential for DSB-induced foci formation of MRE11, are indispensable for HR activity. The table summarizes the relationship between the site of NBS1 mutation and DNA damage response (HR activity, NBS1 foci formation, and MRE11 foci formation). (+): a little or no effect. (–): abrogate the listed function.

ATM-complemented cells, suggesting that ATM might be dispensable for HR repair. As γ-H2AX interacts with NBS1 through the FHA/BRCT domain, we also examined the role of H2AX in HR repair. H2AX-knockout ES cells showed a decrease in HR activity, and the mutation into the acetylated or sumoylated site of H2AX influenced the DSB-induced foci formation and HR activity. Sumoylation of H2AX was confirmed by an *in vitro E. coli* sumoylation system.[33] Furthernore, the repression of acetylation at common sites between H2A and H2AX by a specific inhibitor also decreased IR-induced foci formation and HR activity. These results suggest that the modification of H2AX is related to the recruitment of DSB-related proteins and to HR repair. Taken together, both NBS1 and H2AX could function in HR repair, although ATM, which functionally and physically interacts with NBS1, is dispensable for HR.

4. The role of the Mus81-Eme1 endonuclease in maintenance of genome integrity

The heterodimeric Mus81-Eme1 structure-specific endonuclease plays a role in perturbed replication fork processing and DNA repair by HR. The complex preferentially cleaves nicked Holliday junctions, aberrant replication fork structures, D-loops, and 3'-flap structures, suggesting its roles both upstream and downstream of HR.[34] Dysfunction of Mus81-Eme1 leads to hypersensitivity to a wide range of DNA-damaging agents. In yeast, mus81 mutants are hypersensitive to ultraviolet light, methylmethane sulfonate, camptothecin, and hydroxyurea, suggesting a role for the endonuclease in the rescue of stalled and collapsed replication forks.[35] In contrast, murine and human Mus81 and Eme1 mutant cells are hypersensitive to mitomycin C and cisplatin but not to camptothecin.[36,37] In addition, Mus81-Eme1 has been proposed to play a role in processing spontaneous DNA damage[38]. In this chapter, evidence that the complex is involved in the maintenance of genome integrity is assessed.

An increase in chromosome aberrations represented by breaks, triradials, dicentrics, and fusions is observed in Mus81 and Eme1-deficient mammalian cells.[38,39] Furthermore, the frequency of aneuploidy is increased in these cells. Remarkably, haploinsufficiency of Mus81 or Eme1 also leads to these aberrations, suggesting that the proper biallelic expression of Mus81 and Eme1 is required for the maintenance of chromosome integrity in mammalian cells. Because these aberrations are observed in the absence of exogenous DNA damage, Mus81-Eme1 plays a role in processing spontaneous DNA lesions.

Mus81$^{-/-}$ murine cells accumulate in G2. Phosphorylation of Chk1 is elevated in these cells, indicating that the Chk1-mediated checkpoint is activated in response to spontaneous DNA damage.[37] We examined the mechanisms underlying checkpoint activation using synchronized human HCT116 cells.[38] Both damage-induced Chk1 and Chk2 phosphorylation was increased in Mus81 or Eme1 mutant cells during the S phase. Silencing of ATM reduced the frequency of cells with damage-induced Chk1 or Chk2 phosphorylation, whereas silencing of ATR did not affect the frequency. In addition, phosphorylation of Chk2 was increased in these cells in G2, which was reduced by silencing of ATM. These observations suggest that spontaneous DNA damage generated by Mus81-Eme1 dysfunction activates both the intra-S-phase and G2 checkpoints (Fig. 4).

The p53-mediated checkpoint activation is not observed in Mus81$^{-/-}$ cells in the absence of exogenous DNA damage.[38] However, increased activation of p53 is observed in Mus81$^{-/-}$ cells compared with wild-type cells following mitomycin C treatment.[40] This observation suggests that the p53-dependent checkpoint is activated in response to interstrand cross-linking-induced DNA damage in the absence of Mus81.

Both Mus81$^{+/-}$ and Mus81$^{-/-}$ mice exhibited a profound predisposition to lymphomas and other solid tumors.[39]

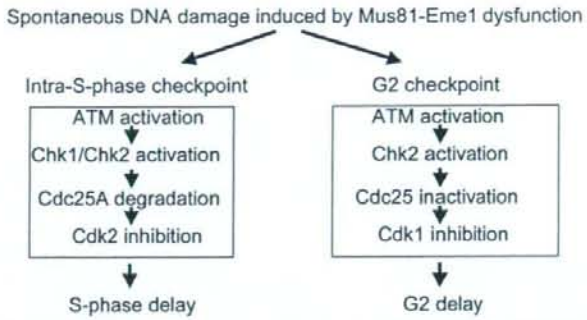


Fig. 4. Checkpoint activation in response to spontaneous DNA damage in HCT116 cells with Mus81 or Eme1 dysfunction.

However, no increased susceptibility of tumor has been observed in another mouse model.[37] It is therefore possible that Mus81-Eme1 dysfunction does not directly lead to tumorigenesis but rather contributes to chromosome instability. Importantly, a recent study has indicated that loss of one allele of Mus81 increases the predisposition of $p53^{-/-}$ mice to sarcoma.[40] This observation suggests that Mus81 may play a role in suppressing sarcoma formation in collaboration with p53.

Thus, accumulating evidence suggests that cellular checkpoints are activated in response to both spontaneous and exogenous DNA damage in cells with Mus81-Eme1 dysfunction. Mus81-Eme1 is therefore likely to play a role in the maintenance of genome integrity in collaboration with multiple checkpoint pathways.

5. FA pathway and homologous recombination repair

Fanconi anemia (FA) is a rare hereditary disorder characterized by progressive bone marrow failure, compromised genome stability, and increased incidence of cancer (reviewed in Wang 2007.[41] FA is caused by genetic defects in altogether 13 genes but this number may further increase in the future. These include genes encoding components of the FA core complex (FancA/B/C/E/F/G/L/M), a key factor FancD2, breast cancer susceptibility protein BRCA2/FancD1, BRCA2's partner PALB2/FancN, BRIP1/FancJ helicase, and just recently discovered FancI. In addition, there are a few gene products that associate with the FA core complex (i.e. FAAP100 and FAAP24 proteins) but without known FA patients lacking these factors.[41]

It has been well known that cells from FA patients display hypersensitivity to DNA crosslinks,[42] and in this regard they seems to resemble cells deficient in HR proteins such as Rad51 paralogs.[43,44] Moreover, they are often mildly sensitive to ionizing irradiation as well. These data may support an idea that basic defects in FA patients could be related to DNA DSB repair. However, until recently, the role played by FA proteins is largely unknown, except for the case of BRCA2, which regulates the central HR protein Rad51.[45] In the DNA damage response, FancD2 and FancI proteins (they form D2-I complex) are targeted to chromatin and forms nuclear foci following their monoubiquitination, a process likely catalyzed by the FA core complex.[41] These foci colocalize at least partially with Rad51 as well as BRCA1.[46] The monoubiquitination is critical for regulating nuclear dynamics of FancD2 (unpublished) as well as tolerance to cisplatin treatment.[47,48] BRCA2/FancD1, PALB2/FANCN, and BRIP1 helicase are not required for FancD2/FancI monoubiquitination, but they should act downstream of, or in parallel to, the core complex-FancD2/FancI pathway.[41]

We planed to examine function of the FA pathway by making knockout cell lines lacking FA proteins in chicken B cell line DT40.[49] The rationale to choose this system is that there are a number of HR assays that could be performed in DT40 cells, and other genetic models such as yeast *S. cerevisiae* do not have a set of FA genes.[41] Our DT40 FA mutant cell lines display similar basic phenotypes. They grow slower than wild type cells, and are hypersensitive to DNA crosslink inducer cisplatin, while radiation sensitivity is quite mild. We first tried to examine whether these mutant cells show defects in HR repair of chromosomal DSB induced by restriction enzyme I-SceI. In this assay, cells that have undergone HR repair form neo-resistant colonies, and the number of the colonies indicates DSB repair activity mediated by HR. We found that *FANCD2*- or *FANCG*-deficient cells are indeed defective in this HR assay.[50,51] Our report was the first to show that the FA pathway is required for normal HR repair. Then we looked at the repaired chromosomal site in *fancd2* cells by Southern blotting, and found that HR repair in this system was compromised not only quantitatively but also qualitatively.[51] The mode of the HR repair was altered such that fraction of long tract gene conversion (LTGC) was decreased from 15% to

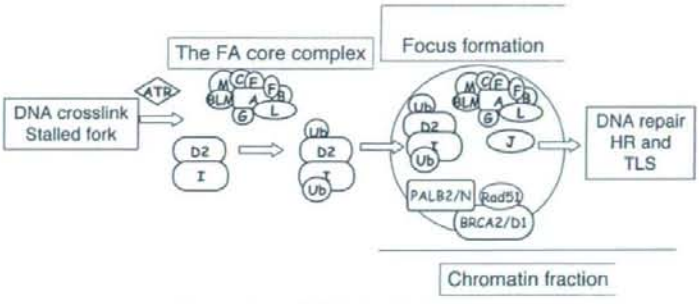


Fig. 5. A simplified view of the FA pathway.

2.7%. Furthermore, ~5% of cells undergo aberrant repair that apparently started with HR but ended by ligation due to non-homologous end joining.[51]

The utility of the DT40 system in HR research is highlighted by the phenomenon "immunoglobulin gene conversion (Ig GCV)". Chicken B lymphocytes diversify its Ig variable gene by GCV mechanism, which depends on Ig transcription, AID expression, and a set of HR factors.[52] DT40 is originated in retrovirally-induced lymphoma in the Bursa of Fabricius, and still continues GCV in *in vitro* culture condition.[53] We found Ig GCV occurs at significantly reduced rate in *fancd2* cells, which is consistent with a role of the FA pathway in HR.[51]

HR repair is proficient mainly during late S to G2 phases in the cell cycle,[54] perhaps because of availability of the template (sister chromatid) and DSB end processing regulated by CDK[55] as well as CtIP protein.[56,57] Therefore we expected that FA protein deficiency should affect DSB repair in those cell cycle phases. Indeed, we found that synchronized *fancd2*-deficient cells display higher radiation sensitivity in late S to G2 phase compared to G1 to early S phases.[51] Kinetics analysis of IR-induced chromosome aberration also supported this notion. Then we looked at IR sensitivity in *ku70/fancg* double knockout cells. In the absence of Ku70 protein, a critical NHEJ factor, DT40 cells are more tolerant to IR than wild type in higher dose range (4–12 Gy),[54] suggesting that presence of Ku may hampers access of HR factors to the broken ends.[58] The double knockout cells are slightly but significantly more IR sensitive than *ku70* single knockout cells (unpublished data), consistent with the role of the FA pathway in HR but not in NHEJ.

We have also analyzed relationship between the classical FA pathway (the core complex-FancD2-FancI pathway) and FancD1/BRCA2.[59] BRCA2 is essential for IR- or MMC-induced Rad51 foci formation but not for FancD2 foci formation, suggesting that the former is not a prerequisite for the latter. Likewise, FancD2 foci formation is not required for Rad51 foci formation. Consistently, DNA damage-induced chromatin loading of Rad51 is normal in cells deficient in FA proteins, raising a possibility that the FA pathway and BRCA2-Rad51 pathway are, at least in their activation phase, independent with each other and in a parallel relationship.[59]

In conclusion, our data clearly demonstrated that the FA pathway participates HR repair (more extensively reviewed in Takata et al. 2006, 2007).[49,60] Interestingly, *BRCA2/FANCC* double knockout cells show similar levels of IR sensitivity with BRCA2 mutant.[59] Taken into account with Rad51 focus and chromatin loading data, this may suggest the FA pathway acts downstream of Rad51. However, further work is needed to draw definite conclusion regarding the function of the FA pathway.

6. UBC13, a ubiquitin E2 conjugating enzyme, plays critical roles in homologous recombination-mediated double strand break repair

Ubiquitylation is involved in DNA repair including nucleotide excision repair, crosslink repair, and postreplication repair (PRR). Rad6/Rad18, a ubiquitin E2/E3 enzyme complex, monoubiquitinates lysine 164 of PCNA, thereby facilitates the loading of translesion polymerases including Polη at blocked forks to resume replication.[61,62] Another E2 enzyme, Ubc13 poly-ubiquitinates PCNA through lysine 63 of ubiquitine (K63) to regulate PRR in yeast. K63 poly-ubiquitination does not appear to involve recognition by the proteasome,[63,64] and its role in damage response has been unclear.

Zhao in Takeda's laboratory recently reported that vertebrate Ubc13 plays a critical role in HR-mediated DSB repair as well as PRR.[65] UBC13$^{-/-}$ DT40 cells show hypersensitivity to a wide range of DNA damaging agents including UV, X-ray, cross-linkers and camptothecin, and exhibit impaired extension of nascent strand over damaged templates, indicating a conserved role for Ubc13 in PRR in eukaryotic species.

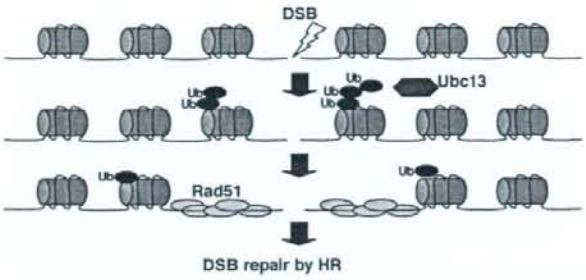


Fig. 6. Ubc13 promotes HR by ubiquitinating proteins at the DSB.

In yeast, Rad18 and Ubc13 are involved in PRR but not HR. Surprisingly, Ubc13$^{-/-}$ DT40 and Ubc13 knockdown human cells show a severe defect in HR as evidenced by a decrease in the frequency of gene targeting and the defective DSB repair of artificial HR substrates. To understand the cause of defective HR, we measured ionizing radiation-induced focus formation. The loss of Ubc13 reduces the focus formation of RPA, a single-strand (ss) binding protein, Brca1, and Rad51 but not that of γ-H2AX or autophosphorylated ATM (ATM^{P1981}). These results suggest that Ubc13 is required for the formation of a single-stranded overhang that is essential for the assembly of Rad51 at DSB ends. To explore a substrate for Ubc13 mediated ubiquitylation, we monitored IR-induced FK2 focus formation, which represents intensive conjugated ubiquitylation at the site of DSB. *UBC13*$^{-/-}$ DT40 cells show virtually no FK2 focus and attenuated mono- and poly-ubiquitylation of γ-H2AX, following IR.[65,66] Thus, H2AX is one of substrates for Ubc13. Presumably, poly-ubiquitylation by Ubc13 modifies local chromatin structure at the site of DSB, and thus increases the accessibility of HR factors including RPA and Rad51. It is of interest whether proteolytic degradation mediated by proteasome and poly-ubiquitylation via lysine 48 (K48) is involved in this Ubc13-dependent pathway. Murakawa et al. analyzed the effect of proteasome inhibitors on DSB repair. Interestingly, treatment of the cells with proteasome inhibitors resulted in phenotypes very similar to those caused by Ubc13 deficiency including the compromised HR and the impaired recruitment of Rad51 and RPA. Thus, the ubiquitin-proteasome system plays a critical role in HR-mediated DSB repair.[67] It should be noted that Ubc13 catalyzes K63-dependent ubiquitylation implicated in signal transduction but not proteosome-mediated degradation. Thus, the relationship between Ubc13 mediated ubiquitylation and proteosome is not necessarily straightforward. Alternatively, it is possible that the proteasome inhibitors reduce free ubiquitin available for conjugation so that cells are unable to perform HR involving ubiquitylation. In summary, Ubc13-dependent ubiquitylation and probably proteolytic degradation are critical for promoting HR, which requires free singlle-stranded DNA tails, because the genome DNA of higher eukaryotic cells is maintained in a highly condensed chromatin folded into a higher order structure (Fig. 6).

7. RAD51 foci and ATM-dependent DNA damage signaling

DSBs induced by ionizing radiation are well known to stimulate the ATM-dependent DNA damage checkpoint pathway.[2] The factors involved in this pathway, such as phosphorylated ATM, form discrete foci at the sites of DSBs, which amplify DNA damage signals.[68] DSBs are repaired by two major repair pathways, NHEJ and HR.[1] Although the factors regulating NHEJ do not form foci in G1, phosphorylated ATM forms foci, and number of which correlates well with the estimated number of DNA double strand breaks. NBS1, involved in HR, has been shown to form foci, and both NBS1 and phosphorylated NBS1 foci are colocalized with phosphorylated ATM foci in G1, S and G2. In contrast to NBS1, little is known about the role of the foci of RAD51, which is the major player in HR and DNA damage checkpoint signalling. The present study examined spatiotemporal relationship between ATM foci and RAD51 foci in normal human diploid cells exposed to X-rays.

By using extensive extraction prior to fixation, we successfully detected RAD51 foci in normal human cells even 30 minutes after X-irradiation with 0.5 Gy (Fig. 7). These foci were mainly observed in the S phase cells, and most of the foci were colocalized with phosphorylated ATM foci. Interestingly, a significant change in the size of phosphorylated ATM was observed, and grown foci were colocalized with phosphorylated NBS1 and phosphorylated BRCA1 foci, while the size of RAD51 foci remained unchanged. Three dimensional analysis revealed that RAD51 foci were included in a part of the large colocalized foci. Thus, it is indicated that phosphorylated ATM foci were created and grew to encircle RAD51 foci, which are the landmarks of chromatin regions processing HR.

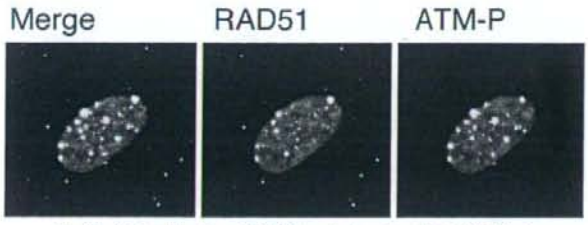


Fig. 7. Colocalization of RAD51 and phosphorylated ATM foci.

These results suggest that the DNA damage checkpoint pathway is activated not only at the sites of DNA damage repaired by NHEJ, but also at the sites processed by HR. In addition, these results indicate that the foci of DNA damage checkpoint factors do not always reflect the sites of DSB repair. Instead, they light up the chromatin regions either directly modified by DSBs, or indirectly altered through DNA repair processes. These secondary changes in the chromatin structure may be involved in amplification of DNA damage checkpoint signals.[69]

8. PERSPECTIVES

Both the HR and NHEJ repair pathways are biologically essential mechanisms for maintenance of chromosome or gene structure in higher eukaryotes. For mammalian immune systems, NHEJ is the central pathway for V(D)J recombination and HR mediates class switching.[70] Because genetic disorders accompanying compromised HR function often presents cancer predisposition, normal HR should be an absolutely error-free repair pathway. Recently, it was reported that generation of DSBs associated with DNA replication stresses such as stalled replication forks closely related to cancer incidences and that these DNA replication-related DSBs are repaired through the HR pathway.[71] This finding suggests the importance of HR repair for cancer prevention. In contrast, failure in regulation of HR often causes chromosomal translocation such as t (7:14) at TCR loci in AT patients,[72] suggesting that the HR pathway also has potential risk of genetic alteration during DSB end processing.

It is still unclear how much NHEJ pathway is error prone. After DNA resection by RAG 1/2, NHEJ proteins in V(D)J recombination somehow 'accurately' join the DNA ends although terminal deoxynucleotidyl transferase inserts additional sequences at a cording joint.[70] This suggests that the majority of DNA ends could be accurately rejoined by NHEJ. One of the reasons why the NHEJ is thought to be error prone is because the chemical structure of radiation-induced DSB ends varies and those ends are often devoid of 5'-phosphate and/or 3'-OH groups. Accordingly, these abnormal ends must be removed by a nuclease for subsequent ligation. This end processing could result in a loss of several bases adjacent to the break point. Establishment of a quantitative assay that enables us to assess both the yield of different types of radiation-induced DSB ends and the efficiency of 'accurate' end processing should be helpful to solve the raised question.

Nuclear foci formation is also a mystery of DNA damage response. It is not well understood, in spite of intensive investigation by many researchers, why such many molecules must localize at the damaged site. It is no doubt that the foci, which are formed immediately after irradiation, must be the exact sites of DNA damage and repair reactions. The majority of the known foci-forming proteins are related to HR pathway whereas none of NHEJ-functioning proteins are reported to form the radiation-induced nuclear foci. Although the phosphorylation foci of DNA-PKcs following DNA damage induction is reported,[73] this may not be *bona fide* nuclear foci formed via relocalization of the protein molecule itself. These observations suggest that the early nuclear foci could be sites of HR-repair.

In contrast to early nuclear foci, what is the biological function of the foci remaining for long time after DNA damage induction? Although it is suggested that these foci are sites of chromatin remodeling, almost all the DSBs disappear within several hours after irradiation. Thus, it is not clear why the chromatin remodeling sites persist long after the completion of DNA repair reaction. Further analysis of the mechanism of protein relocalization and chromatin remodeling would dissolve the mystery.

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