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# Concepts, Labeling Procedures, and Design of Cell Proliferation Studies Relating to Carcinogenesis

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**Chemicals may induce cell proliferation directly as mitogens or indirectly via cell death with subsequent proliferation to replace lost cells. Chemically induced proliferation has been demonstrated to play a role in the carcinogenic process. A wide range of procedures and techniques are currently being used to define the quantitative relationship between the extent and duration of chemically induced cell proliferation and carcinogenic potential in different species and target organs. However, a limited database and nonstandard protocols and procedures for measuring cell proliferation have made it difficult to compare results between laboratories. Comparison of frequencies of S phase between control and treated animals is the most commonly used end point in cell proliferation studies and may be regarded as an indirect indication of a proliferative response.This response can be ascertained as labeling indexes (LI; percentage of cells in S phase) after the administration of the DNA precursor labels (tritiated thymidine; $^3$H-TdR; bromodeoxyuridine, BrdU) or through immunostaining of the endogenous cell replication marker, proliferating cell nuclear antigen (PCNA). Both approaches are applicable to tissue sections. An important issue in the design of experimental studies for measuring LI is determining how and when to investigate proliferative responses in relation to the chemical treatment regimen. Variables to consider when designing cell proliferation studies include the animal's age, chemical dose and method of treatment, choice and dose of label, time and length that the label is administered, and methods of quantitation. Study design considerations depend on the experimental objective. A common approach to characterize the complex relationship of cell proliferation and carcinogenic activity has been to focus on relatively early (less than 90 days) proliferative responses in the target tissue. However, a larger database on the duration and nature of the chemically induced proliferative response under bioassay conditions in the target cell population is required to more clearly establish the role of this end point in the cancer process. In addition, studies must also investigate mitogenic versus cytotoxic induction of cell proliferation in normal and preneoplastic cells and differential toxicity that may provide a preferential growth advantage to spontaneous or chemically induced intermediate or malignant cells.**

## Introduction

Carcinogenesis is a complex process in which normal cellular growth-control genes are altered by several, possibly sequential, mutational events with subsequent clonal proliferation of the resulting precancerous or cancerous cells (*1,2*). Chemical carcinogens may act by inducing mutations and/or altering cellular growth control (*3*). For nongenotoxic carcinogens, the induction of cell proliferation and its effects on the processes of tumor initiation, promotion, and progression appear to be important events in the formation of tumors (*4*). Currently, there is a limited database for chemicals in which induced proliferation has been measured over time under bioassay conditions. The database assessing cell turnover in the developing neoplastic lesions is even more limited. A glossary of terms describing processes related to chemically induced cell proliferation is presented in Table 1.

One class of chemical carcinogens is the genotoxicants. These compounds or their metabolites interact

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**Table 1. Definitions of terms**

| Term | Definition |
|---|---|
| Apoptosis | Physiological single-cell death |
| Bromodeoxyuridine (BrdU) | Thymidine analog used as a labeling agent in cell proliferation studies |
| Cell replication | Production of two daughter cells by the process of replicative DNA synthesis and subsequent cell division |
| Cell turnover | The steady-state rates of cell replication and cell death in a tissue |
| Cytotoxicants | Chemicals that cause cell degeneration and/or cell death |
| Hyperplasia | Increase in the size of tissue through an increase in cell number |
| Hypertrophy | Increase in the size of a tissue through an increase in cell size |
| Labeling index (LI) | Percentage of labeled cells |
| Mitogens | Chemicals that induce hyperplasia with no observable cytolethality |
| Mitotic index (MI) | Percentage of cells undergoing mitosis |
| Proliferating cell nuclear antigen (PCNA) | Endogenous cell cycle-related nuclear protein; auxiliary protein of DNA polymerase δ |
| Pulse labeling | Single administration of a DNA precursor label |
| Pump labeling | Continuous administration of a DNA precursor label via implantation of an osmotic pump |
| Regenerative proliferation | Cell division induced to replace dead cells |
| S phase | Synthesis phase portion of the cell cycle in which nuclear DNA is replicated. During S-phase, DNA precursors such as $^3$H-TdR, and analogues such as bromodeoxyuridine are incorporated into the DNA |
| $^3$H-TdR | Radiolabeled DNA precursor; labeling agent in cell proliferation studies |

with DNA and induce mutations or chromosomal alterations. The observation that most mutagens are also carcinogens is the basis for many current predictive assays and risk assessment models. However, there are also various classes of carcinogens that do not interact directly with DNA; these are frequently referred to as nongenotoxic carcinogens. Subclasses of nongenotoxic agents are mitogens and cytotoxicants. For some nongenotoxic carcinogens, induction of cell proliferation in target tissue appears to be associated with cancer development (*5*). For mitogens and cytotoxicants, differential toxicity and/or growth stimulation may provide a preferential growth advantage to spontaneous or chemically induced initiated or malignant cells. Mutagens are more effective carcinogens at doses that also induce cell proliferation, and mutations may occur secondary to increased cell turnover (*4*). Thus, chemically induced cell proliferation may potentiate the outcomes of both genotoxic and nongenotoxic carcinogens and should be considered in risk assessment (*5*).

Although cell replication is intricately involved in the carcinogenic process, there are rapidly proliferating tissues in the body where cancer is rare. For example, the spontaneous tumor frequency is low in the small intestine, where cells proliferate at a very high rate. In such cell populations, inherent mechanisms must exist to repair genetic damage or eliminate altered cells (i.e., cell death and/or differentiation) before cancer progression can occur. The magnitude of proliferation over that of the normally occurring cellular processes may be important in cancer development. An increased number of critical processes, i.e., mutations, may be required for the induction of cancer in proliferating cell populations as compared to a lower number of critical events in nonproliferating cells or tissues. Thus, just as mutagenicity does not always translate into carcinogenicity, not all chemically induced cell proliferation leads to cancer.

## Chemically Induced Cell Proliferation

Chemically induced proliferative responses may be classified as either mitogenic or cytotoxic. Increased cell turnover may result through chemical interactions with cellular receptors, growth factors, and growth regulatory genes; by causing cell death and subsequent regenerative growth; through interrupting tissue growth control mechanisms such as cell-cell communication; and by inhibiting programmed cell death (apoptosis). Mitogens induce cell proliferation without any detectable cell death, resulting in an increase in the size of the target organ. In contrast, cytotoxicants produce necrosis that is often followed by reparative cell proliferation. Sustained cell proliferation in normal cells produced by a cytotoxicant may increase the frequency of spontaneous mutations that yield neoplastic cells. Growth factor signals regulating regeneration may provide a preferential stimulation to precancerous cells, although the relative proliferative effects of cytotoxic agents on normal and initiated (preneoplastic) or malignant cells have received little attention. Due to the different effects of cytotoxicants and mitogenic agents, their role in the carcinogenic process may be different (*5,6*). A cytotoxicant may provide a selective growth advantage to initiated cells by producing greater cell death in normal cells relative to initiated or neoplastic cells (*7*). The potential for carcinogenic action related to cytotoxicants appears to be secondary to the induced cytolethality and subsequent cell proliferation. It has been suggested that, in the special case of those nongenotoxic chemicals that induce regenerative cell proliferation in the target organ secondary to cytotoxicity, the potential for carcinogenic activity may be decreased below exposures that do not induce a cytotoxic/proliferative response (*6*).

Carcinogenic effects and activity may be different for mitogen-stimulated cell proliferation (*5,8,9*). Mitogens tend to induce a relatively short burst of cell pro-

liferation during chemically induced tissue growth immediately after exposure; this response is followed by a return of cell turnover rates in the normal tissue to baseline levels. Organ size, however, may remain elevated as long as the agent is administered, resulting in an increased number of proliferating cells in which to maintain the increased cell population. Although the mechanism of action of mitogenic carcinogens is unknown, these agents may provide a selective growth advantage to precancerous cells via growth inhibition of "normal" cells, inhibition of apoptosis in precancerous cells, and/or selective proliferative stimulation (*10-13*). Analysis of chemically induced cell proliferation in specific precancerous cell populations, i.e., hepatic foci, is needed to better understand the effect of mitogens on tumor development. Because mitogens may act via cellular receptors, the dose-response issue may be a consideration of the quantitative relationship between receptor occupancy and the triggering of the associated response. Experience with various specific receptor-mediated chemicals demonstrates that a range of exceedingly low doses to very high doses are required to elicit the same effective response. In the case of these agents, tissue- and species-specific responses must be related to carcinogenicity (*14*).

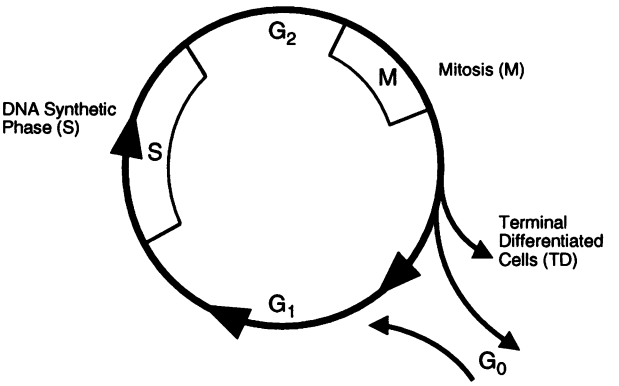


FIGURE 1. Cell cycle. Diagram depicting stages of cell cycle. Multiple control points exist throughout the cell cycle to regulate the entry and progression of the cell in each of the specified stages. During the DNA synthetic phase or S-phase part of the cell cycle, cells synthesizing DNA and will incorporate a specific DNA precursor label such as $^3$H-thymidine and BrdU, or show high expression of proliferation cell nuclear antigen and endogenous cell replication marker. The visualization of these labels and markers allows the determination of the labeling index which is an indirect indication of the cell proliferation response. $G_0$, resting phase; $G_1$ and $G_2$, gap phases in cell cycle between DNA synthetic phase and mitosis.

## Labeling Procedures

Multiple procedures can be used to assay chemically induced cell proliferation. The use of different protocols and procedures makes it difficult to define quantitatively the relationship between chemically induced cell proliferation and carcinogenic potential in different species and target organs. Variables of concern include animal age and species; chemical dose; treatment protocol; choice of label, timing, dose and duration of labeling; and methods of quantitation (*15*).

Comparison of S-phase labeling indexes (LI) between control and treated animals is the most commonly used end point in cell proliferation studies. Because S phase is obligatory to cell replication, its frequency is regarded as an indicator of the cell proliferation response. Chemically induced cell proliferation is typically assessed after the administration of DNA precursor labels ($^3$H-TdR or BrdU) or through the analysis of endogenous cell replication markers such as the proliferating cell nuclear antigen (PCNA; Fig. 1). The ability to detect chemically induced S phase in tissue sections allows for determining proliferative responses in specific target cell populations and correlating these with histopathological changes. Specific techniques for labeling and suggested protocols have been reviewed (*15*).

The most generally used precursor label for DNA synthesis is $^3$H-TdR. Its widespread use in cell proliferation studies is due to its availability in highly specific activity, its specific incorporation into DNA, and the ease of measuring by a variety of techniques. Although high doses of $^3$H-TdR induce perturbations of cell-cycle dynamics, low doses of $^3$H-TdR do not appear to affect cell proliferation analysis (*16*). The most commonly used form of $^3$H-TdR is labeled on the methyl group. In rodent cell-proliferation studies, the typical preparations of methyl-$^3$H-TdR would have specific activities in the range of 25-90 Ci/mM and a concentration of 1 mCi/mL. $^3$H-TdR can be administered to rodents via intraperitoneal injection for use in pulse-label experiments or in osmotic pumps for use in continuous-label studies. Histoautoradiographic techniques are used on tissue sections to identify cells in S phase. Specific technical factors, proper internal controls, and potential problems in the use of $^3$H-TdR and autoradiography have been reviewed (*16,17*). A significant drawback in the use of histoautoradiography is the extended autoradiographic exposure times. As an example, if a rat had been given a $^3$H-TdR solution for 3 days via an osmotic pump at 10 μCi/hr, the autoradiographic exposure period for a liver section would range from 4 to 10 weeks.

BrdU is a thymidine analog that has been used in place of $^3$H-TdR to label a variety of tissues *in vivo*. BrdU-labeled DNA is visualized using immunohistochemical techniques and is as sensitive as $^3$H-TdR for determining chemically induced hepatic and renal cell proliferation (*18*). Advantages of using BrdU are the elimination of the containment problems inherent with use of a radioisotope and the rapid results available by immunostaining that requires hours to days rather than weeks. A disadvantage of the BrdU technique is the lack of a definitive lower-end-cut-off for determining whether a cell is labeled or not. In practice, this concern does not appear to be a significant limitation

for determining LI because detection of BrdU-labeled cells are relatively straightforward to recognize. A greater concern is the recent report of adverse effects of BrdU (*19*), suggesting that BrdU may be toxic, resulting in an enhanced LI. Therefore, dose-related toxicity should be determined for each specific target tissue and experimental protocol before selecting the appropriate methodology. For example, a concentration of greater than 32 mg/mL of BrdU in a 6-day osmotic pump implanted in untreated control rats (10 μL/hr, 150-250 g rat) and mice (1 μL/hr, 18-25 g mouse) was found to increase the hepatocyte cell proliferation response, presumably due to BrdU-induced toxicity (*20*).

Recently, several monoclonal antibodies to endogenous cell-cycle-related nuclear proteins have been developed that can be used to preferentially identify proliferating cells (*21*). PCNA, with peak concentrations seen in the nucleus in late $G_1$/S phase, may prove to be one of the most versatile marker proteins because it obviates administration of the label and may be used to detect proliferating cells in both fixed embedded tissues, and in frozen tissue samples (*22*). Immunohistochemical methods for detecting PCNA in archival study materials also have been reported (23), suggesting use in retrospective cell proliferation analysis of previously studied material. PCNA analysis also has potential to identify the specific cell populations ($G_1$, S, $G_2$, M) that exist in the cell cycle and the pool of active proliferating cells; however, more characterization studies will be required for this purpose (*22*). If feasible, PCNA analysis may lead to quantitation of a chemical's effect on cell population in different stages of the cell cycle, yielding potentially critical information in understanding chemically induced cell proliferation. The relationship between PCNA analysis of proliferation to S-phase analysis as measured by other means have revealed both strong and poor correlations (*24*). In some cases more PCNA immunoreactivity was observed than would be expected as judged by other markers of proliferation. Although the reason for this is unknown, it may be related to PCNA message stability, differences between antibodies used, regulation of PCNA expression by growth factors, and/or masking of PCNA antigenic determinants. At present, the use and proper interpretation of the cell proliferation response as detected by PCNA analysis requires additional studies for clarification and validation.

## Labeling Study Designs

An important issue in the design of experimental studies for measuring LI is determining how and when to investigate the induced proliferative response in relation to the chemical treatment regimen (*15*). The DNA-precursor label for detecting cells in S phase may be administered either by a single pulse injection or continuously for several days via an implanted osmotic pump. The pulse-labeling method gives an indication of the proliferation at a particular point in time. Continuous labeling detects all cells that were in S phase during the period that the pump is in place. The decision to use the pulse or pump label administration in a cell replication study depends on the experimental objective and the turnover rate of the target cell population. For example, it would be inappropriate to use a 6-day pump to assess increased chemically induced cell proliferation in the crypts of the small intestine because all cells would be labeled in the control group (Goldsworthy, unpublished observation). A critical issue in using pulse-label techniques to measure LI is knowing when to sacrifice animals in relation to chemical treatment. A study design using multiple sacrifice times with prior pulse dosing is required to identify a relatively short time course in the induction of cell proliferation and is the method of choice when determining the time course and shape of the cell proliferation curve immediately after partial hepatectomy or chemical exposure. For evaluating the magnitude of replication in a target cell population with a relatively slow turnover rate (i.e., liver, kidney), to minimize diurnal variations, and to provide a greater window of time to detect a proliferative response, the pump-labeling procedure appears to be the method of choice (*15,25*).

In addition to issues of label administration and methodology, there are a number of other critical variables that must be addressed in the design of studies investigating cell proliferation. These factors include treatment regimen, route and dose of chemical exposure, species, strain, sex, age, diurnal variation, diet, environment, target cell population, method of quantitation, and statistical approaches. All of these factors may directly or indirectly impact on cell proliferation data accumulation and interpretation. For this reason, it is necessary to duplicate bioassay conditions in studies that attempt to correlate chemically induced cell proliferation to carcinogenic activity. For example, when evaluating the hepatic proliferation response to the hepatocarcinogens dichlorobenzene (DCB) and furan, we chose the same doses, dosing regimen, animal species, and experimental conditions that produced tumors in the rodent cancer bioassay (*26,27*). The response seen with cytotoxicant agent furan (Fig. 2) and the mitogenic agent DCB (Fig. 3) are shown as examples of retrospective studies to determine the relationship of target tissue proliferation with chemically induced hepatocarcinogenic activity. Experience with studies to date have demonstrated the importance in evaluating a dose, sex, or species that did not develop tumor formation in order to strengthen or weaken the linkage between the observed proliferative response and cancer activity.

## Dose Selection Consideration for Rodent Carcinogenicity Studies

Dose selection is one of the most important and controversial considerations in the design of long-term

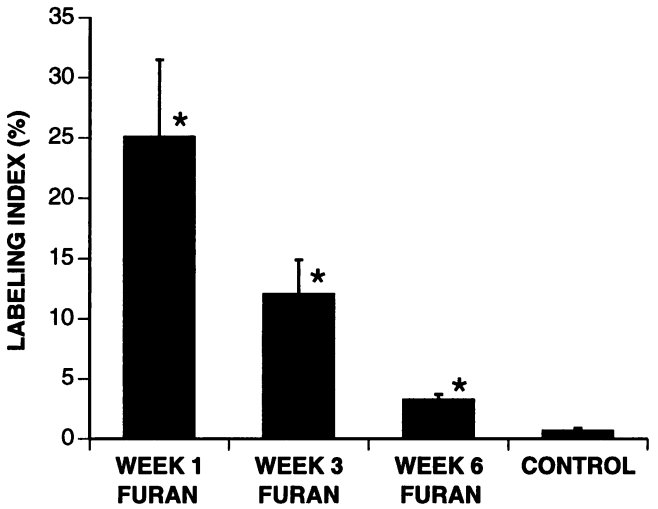


FIGURE 2. Furan-induced hepatocyte cell proliferation. Labeling index in male mice treated for 1, 3, or 6 weeks with the hepatotoxic chemical furan (15 mg/kg body weight) or vehicle (corn oil) under bioassay conditions. Labeling was done with 1 mCi/mL $^{3}$H-TdR administered at 1 μL/hr via an osmotic pump implanted 6 days before necropsy. *Chemical treatment differed significantly from control (Student-Neuman-Keuls' test, $p < 0.05$). Redrawn from Wilson et al. (*27*).

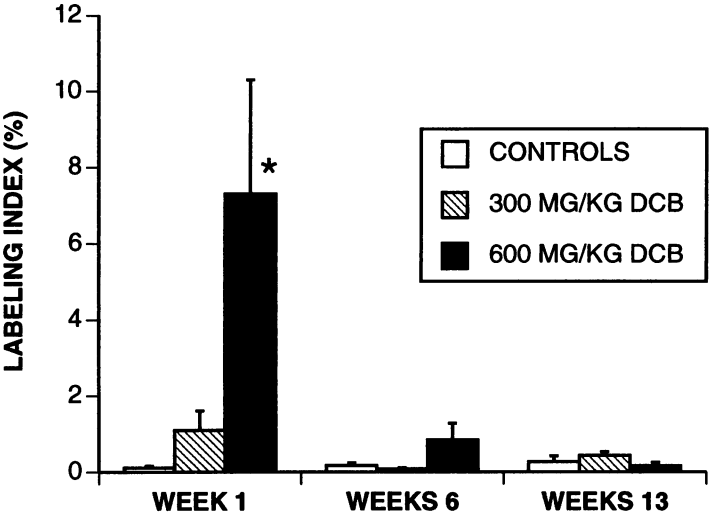


FIGURE 3. Dichlorobenzene-induced hepatocyte cell proliferation. Labeling index in male mice treated for 1, 6, and 13 weeks with the mitogenic chemical dichlorobenzene (600 mg/kg, filled bars; 300 mg/kg, hatched bars) or vehicle control (corn oil, open bars) under bioassay conditions. Labeling was done with 20 mg/mL BrdU administered at 1 μL/hr via an osmotic pump implanted 3 days before necropsy. *Chemical treatment differed significantly from respective control (Student-Neuman-Keuls' test, $p < 0.05$). Redrawn from Eldridge et al. (*26*).

chemical carcinogenesis experiments. With so few animals/dose groups in a bioassay, studies to detect potential carcinogens are frequently designed to include the highest dose of test compound that can be predicted not to alter the animals' longevity from effects other than carcinogenicity (*28*). This dose is referred to as the maximum tolerated dose (MTD). This dose should not cause life-shortening toxicity or more than a 10% decrease in body weight gain. Because MTD determination is typically based on data from prechronic studies, rarely is the optimal exposure level precisely identified (*29*). It is important to avoid, or at least be aware of, situations where high doses of the test chemical compromise the health of animals, overwhelm natural detoxification mechanisms, or yield tumors secondary to excessive organ-specific toxicity. Bioassays are faulted and often required to be repeated at higher doses if no toxicity is seen in the study. Approximately half of the compounds tested by the National Toxicology Program at the MTD are carcinogenic in rodents. Some have raised the concern that many of these responses could be secondary to organ-specific toxicity seen only in the MTD and question the relevance of these observations for predicting carcinogenicity in people at lower levels of exposure (*30*).

Gathering chemically induced cell proliferation data as part of the 90-day study that precedes a cancer bioassay could provide valuable information to aid in the rational selection of both the higher and low doses for long-term studies (*31*). Furthermore, this information might aid in interpreting bioassay results. If one biological property of a chemical to be tested is the induction of cell proliferation, then the shape of the cell proliferation dose-response curve in a target cell population should be one consideration in setting the MTD. Because dose-response curves for a variety of chemically induced alterations (i.e. mutations, proliferation, cell differentiation, gene expression) would be expected to differ from one another, it is essential to integrate all relevant data in selecting the appropriate dosages. It is suggested that selection of at least one dose that does not induce a significant increase in sustained cell proliferation may greatly aid in the interpretation of the results of the bioassay.

Tissues from 90-day studies should be saved in an appropriate manner for retrospective evaluation of proliferative effects at different chemical exposure levels. Including 1 and 3 week groups in addition to 90 days would also be valuable, and may be especially critical when the identification of the target tissue is unknown. Prospective and/or retrospective cell proliferation data may be especially valuable for the nongenotoxicants that induce tumors only when there is chemically induced cytotolethality and sustained regenerative cell proliferation.

## Research Approaches and Future Needs

Many of the current research efforts to better understand the complex relationships between cell prolifera-

tion and carcinogenic activity focus on early tissue proliferative responses. Currently, the relationship between early target tissue proliferation and cancer induction is unclear. A chemical may induce only a short-term alteration in cell proliferation or induce cell proliferation in non-susceptible cells in the target tissue; neither process may be linked to an increased tumor incidence. In addition, proliferative effects early in treatment may not reflect the activity observed after chronic treatment or the proliferative responses within preneoplastic lesions. Thus it cannot be concluded that an early cell proliferative result will equate to an increased risk of cancer. The subacute correlation approach represents a means to identify agents that may be increasing tumor formation through enhanced cell proliferation. Additional data are needed to help clarify the relationship of chemically induced cell proliferation and carcinogenicity.

Investigation of cell-specific proliferation effects through the carcinogenic process is needed to further establish the importance of this end point in chemical carcinogenesis. Quantitation of chemically induced cell proliferation effects is currently available for a limited number of agents. Standard protocols and methodologies for design and measurement of chemically induced cell proliferation are necessary to generate data that may be formulated into a cohesive database. This database will be required to relate the extent, duration, and nature of chemically induced cell proliferation to carcinogenic activity. Cell loss rates must also be studied in conjunction with cell proliferation to accurately determine growth rates of the target cell population. Risk models incorporating cell proliferation data require quantitation of chemicals' effects on the growth and loss of specified target populations as well as the kinetics of those responses. Most important for improved cancer risk assessment is the identification of the mechanism for the chemically induced proliferative responses. Retrospective analysis of cell proliferation and genetic changes in previous studies, coupled with increased mechanistic information from cancer bioassays will yield critical information on the role of chemically induced cell proliferation in the carcinogenic process. Future studies need to investigate mitogenic and cytotoxic induction of differential toxicity and/or growth stimulation mechanisms that may provide a preferential growth advantage to spontaneous or chemically induced precancerous or malignant cells. Cell proliferative responses must be studied in conjunction with oncogene and growth factor expression and mutations, tissue injury and adaption, enzyme induction, and other parameters to ultimately understand complex relationships between cell proliferation and carcinogenesis. Information on the role of chemically induced cell proliferation throughout the cancer process will be valuable in investigations of mechanisms of carcinogenesis, development of assays for nongenotoxic carcinogenic activity, and selection of appropriate risk models.

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