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FIRST GENERATION STOCHASTIC GENE EPISILENCING (STEP1) MODEL AND APPLICATIONS TO IN VITRO CARCINOGEN EXPOSURE

Bobby R. Scott □ Lovelace Respiratory Research Institute

□ A novel first-generation stochastic gene episilencing (**STEP1**) model is introduced for quantitatively characterizing the probability of in vitro epigenetically silencing (episilencing) specific tumor-suppressor-*microRNA* (*miRNA*) genes by carcinogen exposure. Although the focus is mainly on in-vitro exposure of human cells to ionizing radiation, the mathematical formulations presented are general and can be applied to other carcinogens. With the STEP1 model, a fraction f_j of the surviving target cells can have their tumor-suppressor-*miRNA* gene of type j silenced while the remaining fraction, $1 - f_j$ of the surviving cells do not undergo gene episilencing. Suppressor gene episilencing is assumed to arise as a Poisson process characterized with an exponential distribution of episilencing doses with mean d_j . In addition to providing mathematical functions for evaluating the *single-target-gene episilencing probability*, functions are also provided for the *multi-target-gene episilencing probability* for simultaneously silencing of multiple tumor-suppressor-*miRNA* genes. Functional relationships are first developed for moderate doses where adaptive responses are unlikely and are then modified for low doses where adaptation can occur. Results apply to a specific follow-up time t after carcinogen exposure that exceeds the maximum time for the occurrence of an induced episilencing event.

Key Words: radiation, stochastic effects, model, epigenetic, microRNA

INTRODUCTION

Throughout this paper, references to a specific microRNA (miRNA) (e.g., miR-205) are not italicized while references to the related genes (e.g., *miR-205*) are *italicized*. MiRNA-mediated RNA interference has been recognized as a novel mechanism for translational-level regulation of protein expression (Lee and Dutta 2007; Lindsay 2008; Cha *et al.* 2009). MiRNAs are individually encoded by their own set of genes and are an integral part of the genetic program (Chen 2005). They are evolutionary conserved small single-stranded RNAs of 21–25 nucleotides in length that are produced by the endonuclease Dicer from endogenous hairpin-shaped transcripts (Knight and Bass 2001; Gregory and Shiekhattar 2005; Kim 2005; Bartel 2009). One of the strands cleaved by Dicer is incorporated into the RNA-induced silencing complex (RISC) where it induces post-transcriptional, epigenetic silencing (episilencing) of transcripts (RNA) via binding to complimentary target mRNAs (Chen and Meister

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2005; Krol and Krzyzosiak 2006; Weber *et al.* 2007; Flynt and Lai 2008). Protein expression is repressed or the coding message is degraded when miRNAs are bound to the 3'-untranslated regions of target mRNAs (Baek *et al.* 2008; Chan 2011). A single miRNA can regulate numerous target mRNAs and a group of miRNAs can regulate a single transcript in a coordinated manner (Bartel and Chen 2004; Chen and Meister 2005; Lewis *et al.* 2005). Proteins expressed from genes targeted by a particular miRNA tend to be hubs and bottlenecks in the protein interactions (Hsu *et al.* 2008).

The post-transcriptional regulation of gene expression via RNA interference was initially identified in *Caenorhabditis elegans* (Fire *et al.* 1998). Hundreds of *miRNA* genes have been found in the human genome and are responsible for numerous processes that include the control of cell proliferation, apoptosis, tumor formation, immune functions, and an organism's developmental timing (Garzon *et al.* 2006; Kim and Nam 2006; Wu *et al.* 2007; Lindsay 2008; Stefani and Slack 2008; Williams 2008). The miRNAs are also involved in the progression of tumorigenesis from benign to malignant tumors and their regulation depends on a stage of oncogenesis (Cha *et al.* 2009).

A recent study by Tellez *et al.* (2011) revealed that the epithelial-to-mesenchymal transition (EMT) and stem-cell-like properties are associated with epigenetic silencing (episilencing) of specific tumor-suppressor-miRNA genes (*miR-205*, *miR-200b*, and *miR-200c*) during chemical-carcinogen-induced neoplastic transformation of human bronchial epithelial cells (HBEC) repeatedly exposed in vitro. Interestingly, repeated exposures over weeks apparently led to episilencing of most of the target *miRNA* genes among different cells but some residual miRNA expression remained as was also the case in studies by others using normal human fibroblast and gamma rays (Simone *et al.* 2009). The study of Simone *et al.* focused on expression of tumor-suppressor miRNAs let-7a and let-7b. The indicated observations can be explained on the basis of a hypothesized fraction f of target cells that are susceptible to episilencing of the target *miRNA* gene and resistant fraction $1-f$ of cells that do not undergo episilencing of the indicated gene. This might be expected especially if intercellular signaling (e.g., cell community stress response) is involved in the silencing process in which case some cells may be beyond the signaling range.

The activation of the EMT process has recently been implicated as an important step in the metastasis of lung and other tumors (Islam *et al.* 1996; Tellez *et al.* 2011). The process is characterized by changes in several molecular pathways and networks and it appears that the *loss of E-cadherin expression is a critical step that drives this development process in human lung and other cancers* (Tellez *et al.* 2011). The miR-200 family and miR-205 are key determinants of the epithelial phenotype via targeting ZEB1 and

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ZEB2, which has been interpreted to demonstrate that miRNAs can indirectly regulate E-cadherin expression (Gregory *et al.* 2008; Park *et al.* 2008; Tellez *et al.* 2011). ZEB1 and ZEB2 are EMT-inducing transcription factors (Tellez *et al.* 2011). Presently there are no published mathematical models that allow for quantitatively evaluating the carcinogen-induced episilencing probability for tumor-suppressor-*miRNA* genes such as *miR-200b*, *miR-200c*, *miR-205*, *let-7a*, and *let-7b* or for oncogenic *miRNA* genes.

The focus of this paper is on introducing a mathematical model for characterizing carcinogen-induced episilencing of tumor-suppressor-*miRNA* genes in vitro. Model-related mathematical functions and parameters are presented which facilitate characterizing the in vitro episilencing probability per surviving target cell and the average episilencing events per surviving target cell. Initial modeling focuses on moderate doses of carcinogen where adaptive responses are considered unlikely. Results obtained are then modified to allow for low-dose adaptive responses. The modeling results obtained for gene episilencing by moderate doses are used to draw inference about the shape of the dose-response curve for neoplastic transformation in vitro as it relates to carcinogen exposure in circumstances where adaptive responses are presumed not to occur. The modeling framework for moderate doses is outlined in the Methods section and modified in the Discussion section to allow for adaptive responses after low carcinogen doses. Some discussion of the time distribution of tumor-suppressor-*miRNA* gene episilencing is also provided but the distribution is not formally modeled.

METHODS

A target cell with a specified tumor-suppressor-*miRNA* gene of type j (e.g., *miR-200b* or *miR-200c* or *miR-205* or *let-7a* or *let-7b*) is counted as being at risk to be episilenced with probability f_j (which represents the expected fraction of cells that may undergo episilencing). The remaining fraction, $1 - f_j$, of cells is treated as not undergoing episilencing of the target gene of interest irrespective of the carcinogen dose. The episilencing of a specific *miRNA* gene as a result of single or repeated exposure in vitro to a carcinogen is assumed to arise as a realization of a Poisson process which occurs at random time τ_s with distribution $g(\tau_s)$ (not modeled). The *miRNA* gene of interest is therefore modeled as active (producing additional miRNA) or silenced (not producing additional miRNA) at fixed follow-up time t , which is presumed to be long enough for all episilencing events to have occurred.

The MiRNA type of interest is assumed to be produced at a constant rate by a given cell while the *miRNA* gene is activated. When the *miRNA* gene is episilenced at time τ_s , production of new miRNA is assumed to cease for that cell but not for other cells for which their *miRNA* gene is

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still active. For this stochastic episilencing process, the follow-up-time t related probability density evaluated with the carcinogen dose x as the independent variable is exponentially distributed with mean episilencing dose given as $d_j = 1/\alpha_j$. The product $\alpha_j x$ (or ratio x/d_j) gives the average number of episilencing events per cell among the fraction f_j of cells that make up the at-risk subpopulation for follow-up time t . *At-risk (fraction f_j) and not-at-risk (fraction $1-f_j$) cells are presently assumed equally sensitive to cell killing, so that cell killing is not modeled. Rather, the episilencing probability per surviving cell is modeled.* This relates to what is observed experimentally (i.e., episilenced genes among surviving cells). The temporal pattern of changes in miRNA molecules is not modeled and results presented relate to a fixed follow-up time t as already indicated. Temporal changes will be addressed in future research. This includes modeling the distribution of times to *miRNA* gene episilencing.

The probability per surviving cell of a carcinogen-induced episilencing of a specified tumor-suppressor-*miRNA* gene of type j (i.e., *single-target episilencing probability*) is given by the exponential function $\psi_j(x) = f_j[1 - \exp(-x/d_j)]$. The product $f_j[1 - \exp(-x/d_j)]$ represents the joint probability of a surviving cell being in the at-risk group (probability, f_j) and also having its target *miRNA* gene episilenced (probability, $[1 - \exp(-x/d_j)]$), given exposure to a dose x of carcinogen and fixed follow-up time t . The probability $1 - \exp(-x/d_j)$ was derived based on $\exp(-x/d_j)$ being the probability per surviving cell that gene episilencing does not occur (i.e., the probability for zero episilencing events when the mean is $\alpha_j x = x/d_j$). The expected distribution of the number of gene episilencing events at follow-up time t per cell when the average is x/d_j can be generated using the Poisson probability mass function. The mean number of episilencing events per cell can be less than or greater than 1, depending on the dose of carcinogen. At present, the specific silencing events are not identified but could include gene promoter hypermethylation and/or specific histone modifications.

All *miRNA* genes that have been linked to epigenetic regulation are closely associated with *CpG* islands (Saito *et al.* 2006; Brueckner *et al.* 2007; Lujambio *et al.* 2007; Weber *et al.* 2007). While most of *CpG*-rich retroelement and repeat sequences are strongly methylated, most gene-associated *CpG* islands are not methylated in normal human cells. The pattern changes during tumorigenesis as tumor cells acquire altered methylation profiles. Tumor-specific methylation patterns are characterized by both hypermethylation and hypomethylation. *These alterations bring about stable changes in gene expression and are thus functionally equivalent to classical genetic mutations* (Weber *et al.* 2007). However, because of the observation that epigenetically silenced tumor-suppressor genes can be activated while in a fully methylated state, methylation of *miRNA* genes should not be interpreted as a mechanism for permanent episilencing (Pruitt *et al.* 2006;

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Weber *et al.* 2007). Whether or not high-level-stress-related intercellular signaling is important for this form of *miRNA* gene activation is unknown. We will investigate this possibility in our future research.

The probability for simultaneous episilencing of multiple tumor-suppressor-*miRNA* genes of types 1, 2, ..., n (i.e., *multitarget episilencing probability*) by follow-up time t is evaluated as the product $\psi_1(x)\psi_2(x)\dots\psi_n(x)$, which is indicated by $\psi_{1,2,\dots,n}(x)$. Subtracting $\psi_{1,2,\dots,n}(x)$ from 1 yields the complimentary survival function $S_{1,2,\dots,n}(x)$. The related *multitarget episilencing hazard* $H_{1,2,\dots,n}(x)$ is equal to “-1” times $\ln[S_{1,2,\dots,n}(x)]$. Taking the derivative of $\psi_{1,2,\dots,n}(x)$ with respect to the dose x gives the associated probability density function $\Theta_{1,2,\dots,n}(x)$, which is useful for modeling other effects (EMT, occurrence of stem-like cells, neoplastic transformation) that are related to the simultaneous episilencing of an at-risk set of tumor-suppressor-*miRNA* genes. This additional modeling will be addressed in future research.

The modeling framework introduced in this section characterized the main features of the first generation **stochastic gene episilencing (STEP1)** model as it relates to moderate doses where adaptive responses are unlikely. The results presented also apply to low doses when adaptive responses do not occur (e.g., are inhibited). The STEP1 model which focuses on tumor-suppressor-*miRNA* genes is discussed in more detail in the Results section. As with most if not all first generation models, the STEP1 model has deficiencies that are discussed below which limit the dose range over which it may apply.

RESULTS

STEP1 Model Attributable Risk and Related Functions

The average number of carcinogen-induced episilencing events per cell for a tumor-suppressor-*miRNA* gene of type j and follow-up time t when cells are exposed in vitro to a non-adaptive-response-invoking dose x of carcinogen is given under the STEP1 model by the single-target gene episilencing hazard (representing the cumulative hazard for target-gene silencing):

$$H_j(x) = \alpha_j x = x/d_j. \quad (1)$$

Equation 1 is assumed to be valid for both low and moderate doses when adaptive responses do not occur (e.g., are inhibited) *but not necessarily for high doses based on reasons provided in the Discussion section*. The dose x can be defined in a variety of ways (e.g., exposure time, number of repeated exposures, concentration-time product for a chemical carcinogen). For radiation exposure, the dose can be energy deposited in the target cell population divided by the mass of cells (i.e., the absorbed dose)

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and therefore can have units such as milligray (mGy), gray (Gy), etc. Throughout this paper the representation involving the parameter d_j will be mainly used (an unconventional approach) as this facilitates derivation of the analytical solutions that are presented, related to the probability density functions for carcinogen-induced episilencing of a given tumor-suppressor-*miRNA* gene. The related single-target episilencing probability per surviving cell for the *miRNA* gene of type j among the types at risk is given as a function of $H_j(x)$ by the following follow-up time t related equation:

$$\psi_j(x) = f_j [1 - \exp(-H_j(x))] . \quad (2)$$

Equation 2 does not include spontaneous episilencing events and therefore represents the *attributable episilencing risk*. Multiplying $\psi_j(x)$ by the number $c(x)$ of surviving cells at follow-up time t gives the expected number of surviving episilenced cells (those cells with the target gene silenced) associated with the dose x . This number can be presumed to have a binomial distribution with mean $c(x)\psi_j(x)$ and variance $c(x)\psi_j(x)(1-\psi_j(x))$. The related single-target episilencing probability density function for the at-risk fraction f_j of cells is given by the following not-normalized equation:

$$\phi_j(x) = d\psi_j(x)/dx = f_j \exp(-x/d_j)/d_j . \quad (3)$$

For simultaneous silencing of two different target tumor-suppressor-*miRNA* genes (indicated by subscripts 1 and 2), the corresponding equation for the two-target-episilencing probability per surviving cell (an attributable risk) after exposure to a dose x of carcinogen is given as follows:

$$\psi_{1,2}(x) = \psi_1(x)\psi_2(x) . \quad (4)$$

The corresponding two-target episilencing hazard is given by the following:

$$H_{1,2}(x) = -\ln[1-\psi_{1,2}(x)] . \quad (5)$$

The corresponding not-normalized, two-target episilencing probability density is given by the following:

$$\Theta_{1,2}(x) = d\psi_{1,2}(x)/dx = f_1 f_2 [\phi_1(x) + \phi_2(x) - \phi_{1,2}(x)] , \quad (6a)$$

where,

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$$\phi_{1,2}(x) = (d_1 + d_2)\phi_1(x)\phi_2(x). \quad (6b)$$

For simultaneously silencing any n (> 2) multiple targets of tumor-suppressor *miRNAs* (different genes), the corresponding multitarget episilencing probability per surviving cell (an attributable risk) is given by the following equation:

$$\psi_{1,2,\dots,n}(x) = \psi_1(x)\psi_2(x)\dots\psi_n(x). \quad (7)$$

The corresponding multitarget episilencing hazard is given by the following:

$$H_{1,2,\dots,n}(x) = -\ln[1-\psi_{1,2,\dots,n}(x)]. \quad (8)$$

The corresponding equation for the not-normalized, multitarget episilencing probability density can be obtained using the following iterative equations (9b form):

$$\Theta_{1,2,\dots,n}(x) = d[\psi_{1,2,\dots,n}(x)]/dx = d[\psi_n(x)\psi_{1,2,\dots,n-1}(x)]/dx, \quad (9a)$$

or

$$\Theta_{1,2,\dots,n}(x) = \psi_n(x)\Theta_{1,2,\dots,n-1}(x) + \psi_{1,2,\dots,n-1}(x)\phi_n(x). \quad (9b)$$

For example, for $n = 3$ (e.g., for simultaneous silencing of *miR-200b*, *miR-200c*, and *miR-205*), one gets the following solution:

$$\Theta_{1,2,3}(x) = f_1f_2f_3[\phi_1(x) + \phi_2(x) + \phi_3(x) - \phi_{1,2}(x) - \phi_{2,3}(x) - \phi_{1,3}(x) + \phi_{1,2,3}(x)]. \quad (10a)$$

where,

$$\phi_{2,3}(x) = (d_2 + d_3)\phi_2(x)\phi_3(x) \quad (10b)$$

$$\phi_{1,3}(x) = (d_1 + d_3)\phi_1(x)\phi_3(x) \quad (10c)$$

$$\phi_{1,2,3}(x) = (d_1d_2 + d_2d_3 + d_1d_3)\phi_1(x)\phi_2(x)\phi_3(x). \quad (10d)$$

The normalization factor for Equation 10a is $f_1f_2f_3$. One can also calculate the expected fold change in the expressed tumor-suppressor miRNAs of type j at follow-up time t as a result of carcinogen exposure using the STEP1 model. Assuming the expressed miRNA of interest at

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fixed follow-up time t is proportional to the number of surviving cells with a functional *miRNA* gene, the expected fold change in the expressed miRNA can be calculated as follows:

$$\text{Fold change} = N_j[f_j \exp(-\alpha_j x) + (1 - f_j)] / N_j = f_j \exp(-\alpha_j x) + (1 - f_j). \quad (11)$$

Here N_j is the average tumor-suppressor miRNA count (number per cell) for unexposed (control) cells for the type of interest which may be on the order of several thousands per cell (Allawi *et al.* 2004). However, for this calculation, N_j is present in both the numerator and denominator and cancels out. *In vivo* research results suggest that N_j can be strongly influenced by the cellular environment (Kuchen *et al.* 2010); however, with the STEP1 model these influences affect both the numerator and denominator in Equation 11 and therefore cancel out.

Application of the STEP1 Model to Episilencing of Specific *miRNA* Genes

Here the initial focus is on the tumor-suppressor-*miRNA* genes *let-7a* and *let-7b* for which there is some information that allows estimating STEP1 model parameters for *in vitro* gamma-ray exposure of normal human fibroblast to moderate radiation doses. Data of Simone *et al.* (2009) indicated about a 60% reduction in *let-7a* expression after *in vitro* exposure of normal human fibroblast to a gamma-ray dose of 1 Gy and about a 70% reduction after a dose of 3 Gy with no additional reduction after a dose of 5 Gy. The data implicates a value for f_1 of about 0.7 and a value of parameter α_1 of about $-\ln[(0.4-0.3)/0.7]/1 \text{ Gy} \approx 2/\text{Gy}$ (based on equation 11). The corresponding estimate for d_1 is 0.5 Gy. For a dose of 10 Gy, the fold-change data were inconsistent with the STEP1 model in that only mild episilencing of *let-7a* (about a 25% reduction in expression) was implicated suggesting that the model's reliability may be limited to doses much less than 10 Gy. This would be expected if cell survival curves were similar for the at-risk (fraction f_j) and not-at-risk (fraction $1-f_j$) cells for low and moderate doses but diverged after a massive dose such as 10 Gy (10,000 mGy) with the cells at risk to episilencing being more sensitive. A dose of 10 Gy would be expected to be highly cytotoxic invoking strong intercellular signaling that may alter miRNA gene episilencing profiles. The high-dose issue will be addressed in future research. Results presented here are restricted to the range 0 to 5 Gy and it is initially assumed that radiation adaptive responses do not occur at low doses. An approach to address radiation adaptive responses at low doses is presented in the Discussion section which allows for relaxing the indicated assumption.

Figure 1 shows the simulated episilencing hazard ($H_1(x) = 2x$) for *let-7a* for *in vitro* exposure of normal human fibroblasts to gamma rays based on the above parameter values. As indicated, the results were derived

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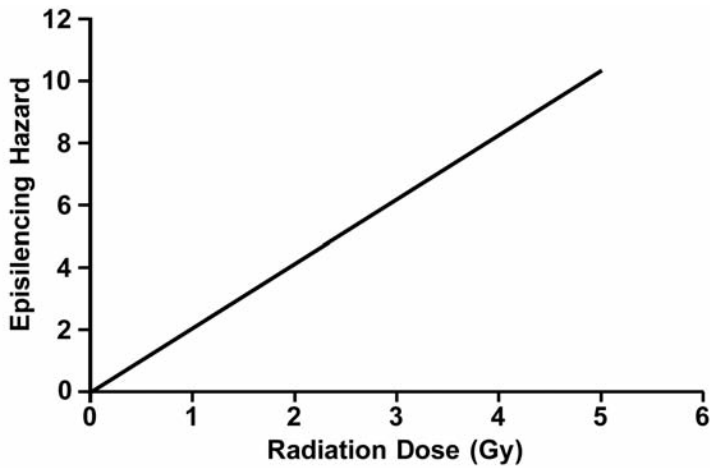


FIG. 1. Estimated episilencing hazard for the *let-7a* for normal human fibroblast exposed in vitro to gamma rays based on data presented in Simone *et al.* (2009) and on the STEP1 model. Adaptive responses were assumed not to occur at low doses.

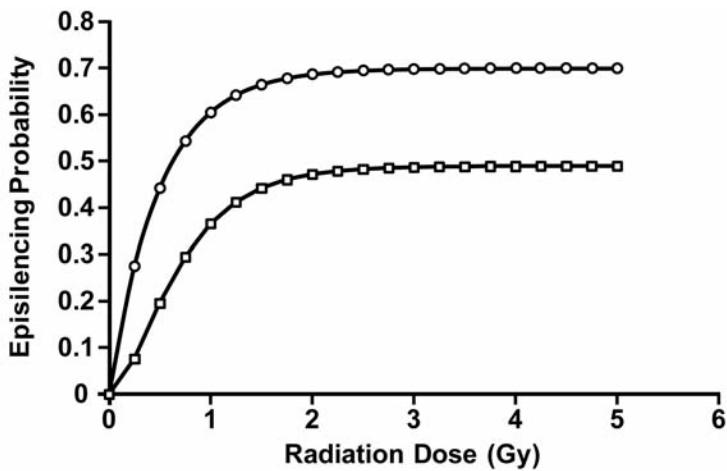


FIG. 2. Open circles: estimated single-target (*let-7a* gene) episilencing probability (attributable risk) based on data of Simone *et al.* (2009) for in vitro gamma-ray exposure of normal human fibroblast and on STEP1 model ($f_1 = 0.7$, $\alpha_1 = 2/\text{Gy}$). Open squares: corresponding results for the two-target (*let-7a* and *let-7b* genes) episilencing probability (attributable risk) based on data of Simone *et al.* (2009) and the STEP1 model ($f_1 = f_2 = 0.7$ and $\alpha_1 = \alpha_2 = 2/\text{Gy}$). Data points indicate doses where calculations were carried out. Adaptive responses were assumed not to occur at low doses.

assuming adaptive responses did not occur after low doses. Figure 2 shows the corresponding calculated single-target episilencing probability ($\psi_1(x) = 1 - \exp(-2x)$) for *Let-7a*. The study of Simone *et al.* (2009) demonstrated a somewhat similar dose-response profile for *let-7a* and *let-7b* expression as a function of the gamma-ray dose. Assuming the same parameters assigned to α_1 and f_1 for *let-7a* also apply to *let-7b*, i.e., $\alpha_1 = \alpha_2 = 2/\text{Gy}$ and

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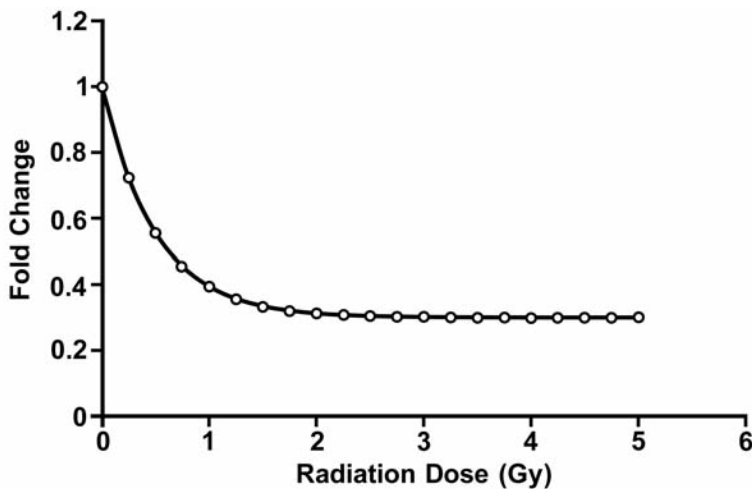


FIG. 3. STEP1 model-based simulated fold change (reduction) in expressed let-7a after in vitro exposure of normal human fibroblast to gamma rays based on the Equation 11 ($f_1 = 0.7$, $\alpha_1 = 2/\text{Gy}$). Data points indicate doses, where calculations were carried out. Adaptive responses were assumed not to occur at low doses.

$f_1 = f_2 = 0.7$, then the calculation of the joint probability of episilencing both tumor-suppressor-*miRNA* genes is according to Equation 4. Results obtained are also presented in Figure 2.

Figure 3 shows the simulated fold changes for let-7a expression based on Equation 11 and the indicated model parameter assignments. The results are consistent with data of Simone *et al.* (2009) in that there is a calculated 60% reduction in let-7a expression after a dose of 1 Gy and a 70% reduction after a dose of 3 Gy with no additional reduction after a dose of 5 Gy. Note the gradient of responses even though gene episilencing is modeled as either occurring or not occurring at the individual cell level. Thus, *experimental data for fold changes in gene expression cannot inform as to whether the underlying biology at the cellular level is stochastic (gene active or episilenced in a given cell) or deterministic (varying degrees of gene episilencing in a given cell)*. Similar dose-response curve shapes (although more dramatic, possibly due to repeated exposures being used) were reported by Tellez *et al.* (2011) for miR-200b and miR-200c expression for chronic in vitro combined exposure of HBEC to the smoking-related carcinogens methylnitrosourea (MNU) and benzo(a)pyrene-diol-epoxide (BPDE). The data are suggestive of a value of f_1 of about 0.9, suggesting that about 10% of the HBEC did not undergo episilencing events for the *miR-200b* and *miR-200c* genes.

The STEP1 model along with an added assumption about multiple *miRNA* gene participation in transformation can also be used to compare the findings of Damiani *et al.* (2008) related to MNU/BPDE-induced neoplastic transformation of HBEC in vitro and the findings of Tellez *et al.* (2011) for the same cells and carcinogens related to the *miRNA* gene

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episilencing. Tellez *et al.* (2011) investigated the occurrences of EMT and stem-like cells using in vitro neoplastic transformation assay previously established (Damiani *et al.* 2008) as well as two already transformed cell lines (HBEC1 from a smoker without lung cancer; HBEC2 from a smoker with lung cancer). Tellez *et al.* (2011) found that a *four-week MNU/BPDE exposure of immortalized HBECs induced a persistent, irreversible, and multifaceted de-differentiation process marked by EMT and the emergence of stem-cell-like properties*. EMT occurrence was epigenetically driven, initially by chromatin remodeling through H3H27me3 enrichment and later by ensuing DNA methylation to maintain silencing of tumor-suppressor-*miRNA* genes (*miR-200b*, *miR-200c*, and *miR-205*), which are implicated in the de-differentiation program in HBEC and also in primary lung tumors. Expression of the *miRNAs* regulating EMT (i.e., *miR-200b*, *miR-200c*, *miR-205*) was significantly reduced at 4 weeks and also in the transformed HBEC1 and HBEC2 cells.

Assuming that simultaneous episilencing of *miR-200b*, *miR-200c*, and *miR-205* are necessary and sufficient for initiating the EMT (*emt*) program with probability Ω_{emt} and subsequent stem-like-cell (*stem*) emergence with probability Ω_{stem} , and subsequent neoplastic transformation (*trans*) with probability Ω_{trans} would implicate cubic dose-response curves for the hazard functions $H_{emt}(x)$, $H_{stem}(x)$, and $H_{trans}(x)$ for the respective effects at low to moderate doses. Evidence for a cubic dose-response for $H_{trans}(x)$ for the induced transformation of HBECs is presented in the Discussion section.

DISCUSSION**Predicting the Shapes of Dose-Response Relationships for Specific Stochastic Biological Effects Based on the STEP1 Model**

The STEP1 model was applied as indicated above to the moderate dose radiation data of Simone *et al.* (2009). Because the model was consistent with the data for moderate radiation doses, it can be used to make predictions about the expected effects of tumor-suppressor *miRNA* episilencing by such doses of a carcinogen and also by low doses when adaptive responses do not occur. It can be stated with some confidence that for any stochastic (characterized by a defined probability) biological effect which is caused by the episilencing of any one among a set of tumor-suppressor-*miRNA* genes that the hazard function for that effect would be expected to be a linear function of the carcinogen dose for the range of doses considered with a slope that increases as the number of *miRNAs* in the at-risk set increases. Where episilencing of two specific tumor-suppressor-*miRNA* genes is causal (i.e., both necessary and sufficient) for the effect, then the hazard function for that effect would be expected to be a quadratic function of carcinogen dose for the range of doses considered.

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Where the episilencing of three specific tumor-suppressor-*miRNA* genes is causal for the effect, then the hazard function for that effect would be expected to be a cubic function of carcinogen dose for the range of doses considered. Where episilencing of n (>3) specific tumor-suppressor-*miRNA* genes is causal for the effect, then the hazard function for that effect would be expected to be proportional to x^n for the range of doses considered. These predictions apply to low doses only in circumstances where adaptive responses do not occur.

For dose-response data for which there is no evidence for an adaptive response, a plausible value of n can be evaluated by plotting the hazard function (adjusted for spontaneous effects) for the effect of interest vs. x , then vs. x^2 , then vs. x^3 , etc. and conduct separate linear regressions (with zero intercept) against these different variables (x, x^2, x^3, \dots). The results yielding the highest correlation (adjusted) would be a plausible value of the integer n for x^n . This has been done to obtain Figure 4 for the neoplastic transformation hazard $H_{trans}(x)$ for HBEC1 cells after repeated in vitro exposures to the carcinogenic mixture MNU/BPDE, based on data reported by Damiani *et al.* (2008). The data did not show any evidence of an adaptive response occurring. The independent variable x was the number of weeks of carcinogen exposure (Figure 5). Thus $x = \text{weeks}$, $x^2 = \text{weeks}^2$, etc. For $n = 1$, R^2 (adjusted) = 0.41; for $n = 2$, R^2 (adjusted) = 0.48; for $n = 3$, R^2 (adjusted) = 0.49; for $n = 4$, R^2 (adjusted) = 0.42; for $n = 5$, R^2 (adjusted) = 0.33. As reflected in Figure 3, $n = 3$ is considered a plausible choice for these data ($n = 2$ and 4 are also plausible) and is consistent with simultaneous episilencing of miR-200b, miR-200c, and miR-205 (i.e.,

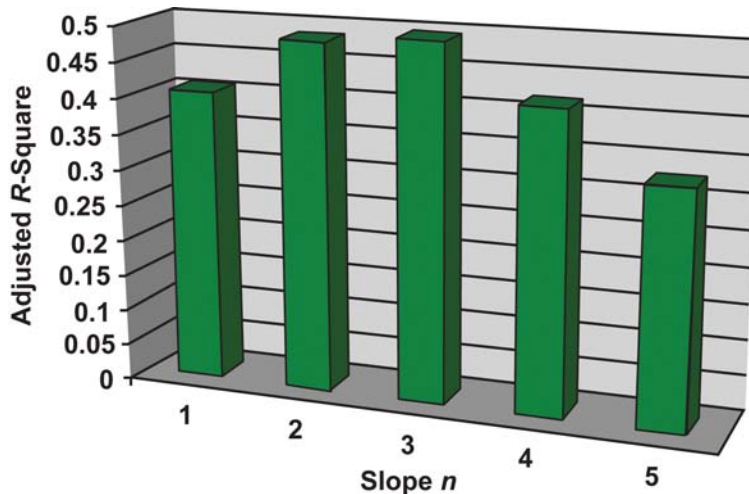


FIG. 4. Search results for finding a plausible value for the slope n for the neoplastic transformation hazard $H_{trans}(x) = ax^n$ (parameter a) for MNU/BPDE exposed HBEC1 cells in vitro based on data from Damiani *et al.* (2008). The vertical axis gives values of the adjusted correlation coefficient when experimental data (presented in Figure 5) for $H_{trans}(x)$ was regressed against x, x^2, x^3, x^4 , and x^5 , where x is the number of weeks of exposure to the carcinogenic mixture.

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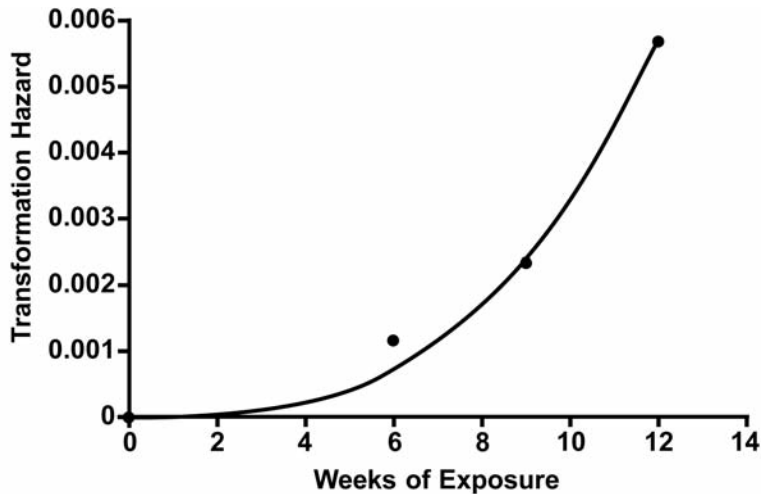


FIG. 5. In vitro neoplastic transformation hazard for MNU/BPDE exposed human bronchial epithelial cells (HBEC1) for normal cells derived from a smoker without lung cancer. The data are based on Damiani *et al.* (2008) with the transformation hazard calculated as $-\ln(1-\text{transformation frequency})$. The smooth curve is based on the hazard function equation $H_{trans} = 3.3 \times 10^{-6} x^3$, where x is the number of weeks of exposure to the carcinogenic mixture. These results suggest the episilencing of 3 target *miRNAs* (possibly *miR-200b*, *miR-200c*, and *miR-205*) as possibly being causal for neoplastic transformation. Adaptive responses were assumed not to occur.

three target genes) as being *possibly causal* for the subsequent neoplastic transformation of HBEC1 cells as was implicated by the study of Tellez *et al.* (2011). Thus, the notion that simultaneous episilencing of *miR-200b*, *miR-200c*, and *miR-205* are possibly causal for entering the neoplastic transformation pathway seems plausible but more definitive research is needed before a firm conclusion can be made.

Relative Importance of Tumor-Suppressor-Gene Episilencing and Mutations

Mutation induction is known to be important for neoplastic transformation and for cancer induction by moderate and high doses of carcinogens. *For exposure to carcinogens at the low end of the known carcinogenic dose range, miRNA episilencing is orders of magnitude more likely than mutation induction.* This finding is based on the assumption that the hazard functions (adjusted for spontaneous effects) for both gene episilencing and mutation induction by gamma rays is linear-non-threshold with the slope for episilencing being > 1 (Figure 1) and for mutation induction being orders of magnitude smaller for mammalian cells when the dose is in Gy (Grosovsky and Little 1985).

Possible Impact of Differential Cell Survival at High Doses

The STEP1 model at present does not directly address cell survival. This is because it was assumed that cells at risk to episilencing and those

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not at risk are equally sensitive to being killed. This assumption allows the modeling to be focused on the episilencing probability per surviving cell. The experimental researcher reports miRNA expression fold-change data for surviving cells so there is no need to model cell survival so long as all target cells are presumed equally sensitive. *Future research will address the possibility of different survival curves for cells that are sensitive and those that are resistant to miRNA silencing.*

Occurrence of Adaptive Responses at Low Doses

There is hard evidence that the miRNA expression response to radiation exposure is quite different for high and low radiation doses (Cha *et al.* 2009), possibly related to different biological signaling pathways being involved after low and high doses. There is growing evidence that low but not high doses of low-linear-energy (LET) transfer radiation such as X and gamma rays stimulate adaptive-response signaling pathways (e.g., DNA repair and protective apoptosis that selectively eliminates aberrant cells) (Scott *et al.* 2009). For in vivo exposure of mammals, low doses of low-LET radiation can also stimulate anticancer immunity while high doses are immunosuppressive (Sakai *et al.* 2003; Liu 2007) which can lead to a hormetic dose-response relationship for cancer induction.

MiRNAs play an important role in regulation of DNA repair, apoptosis, and immune functions (Chen 2005; Lindsay 2008; Crosby *et al.* 2009). For example, Lindsay (2008) points out that recent publications have provided compelling evidence that a variety of miRNAs are involved in the regulation of immunity, including the development and differentiation of B and T cells, proliferation of monocytes and neutrophils, antibody switching and the release of inflammatory mediators. *Since low-dose radiation stimulates anti-cancer immunity (an adaptive response) and high doses are immunosuppressive, differential episilencing of immune-system-related, tumor-suppressor-miRNA genes are hypothesized.* Our future research will investigate this possibility.

Cha *et al.* (2009) looked at the expression of miRNAs isolated from IM9 human B lymphocytes exposed in culture to low (50 mGy) and high (10,000 mGy) gamma-ray doses. The 50 mGy dose caused *decreased expression* (an apparent adaptive response) of the following 13 miRNAs whose expression did not decrease after the high radiation dose: hsa-let-7f-2, hsa-miR-19a, hsa-miR-106b, hsa-miR-376a, hsa-miR-16-1, hsa-miR-23a, hsa-miR-18, hsa-23b, hsa-miR-155, hsa-miR-106a, hsa-miR-17-5p, hsa-miR-21, and hsa-miR-20. In contrast, the 10,000 mGy dose *increased* expression of the following 5 miRNAs that were *not increased* by the low dose: hsa-miR-324-3p, hsa-miR-238, hsa-miR-187, hsa-miR-99b, and hsa-miR-236. These results suggest that the responses to the low dose (50 mGy) may be a mild-stress response involving intercellular communications that relate to an adaptive response. The next section presents an approach to addressing adaptive responses.

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Approach to Addressing Adaptive Responses at Low Doses

The STEP1 model as formulated above does not address tumor-suppressor-*miRNA* gene changes related to an adaptive responses after a low dose of carcinogen. A more complex modeling scheme is needed to account for adaptive responses. In addition to use of the probability $\psi_j(x)$ (attributable risk), a benefit function, $B(x)$, should also be used (Scott 2011a). $B(x)$ represents the probability of occurrence of an adaptive response. Such responses are considered to be **epigenetically regulated cell-community-wide (epicellcom)** responses that involve intercellular and intracellular communications (Scott 2011b). However, in addition to $B(x)$, a second probability is also needed, namely the probability that a specific benefit of interest will occur by follow-up time t , given that an adaptive response has been mounted. This second probably has been represented by what is called the protection factor (*PROFAC*). For a tumor-suppressor-*miRNA* gene of type j that is episilenced at follow-up time τ_s , a benefit would be its epigenetic activation (*epiactivation*) by follow-up time $t > \tau_s + \tau_a$, where τ_a is the random post silencing time to epiactivation (i.e., reactivation). In such cases, $PROFAC_j$ can be used to represent the conditional probability of *miRNA* gene epiactivation by follow-up time t , given that an adaptive response has been mounted as a result of exposure to a dose x of carcinogen. The product $B(x)PROFAC_j$ would then represent the joint probability of a mounted adaptive response and epiactivation of the indicated silent *miRNA* gene by follow-up time t . The distribution of the joint times $\tau_s + \tau_a$ can be obtained by convoluting $g(\tau_s)$ and $q(\tau_a)$ where $g(\tau_s)$ and $q(\tau_a)$ are the related distribution functions (probability densities) for τ_s and τ_a , respectively (Scott 2011b). This will be addressed in future research. Because the *miRNA* gene silencing and subsequent reactivation are stochastic events at the individual cell level, $g(\tau_s)$ and $q(\tau_a)$ are continuous functions of time.

$B(x)$ takes on values > 0 only over the dose range (hormetic zone) where adaptive responses occur. For this range, rather than focusing only on the risk function $\psi_j(x)$, the focus should instead be on the corresponding probability $P_j(x)$ of spontaneous or induced tumor-suppressor gene episilencing where

$$P_j(x) = [1 - B(x)PROFAC_j][1 - \exp(-H_j(x) - H_{j,0})]. \quad (12)$$

The parameter $H_{j,0}$ accounts for spontaneous episilencing. The product on the right hand side gives the episilencing probability when spontaneous occurrences and an adaptive response are both accounted for. When $H_{j,0} = 0$ (no spontaneous occurrences) and $B(x) = 0$ (no benefit), then $P_j(x) = \psi_j(x)$ (the attributable episilencing risk). Because $B(x)$ is expected to have an inverted U-shape (Scott 2011a), the dose-response curve for the indicated product is expected to be hormetic.

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At low doses where $H_j(x) \ll H_{j,0}$ and $B(x) > 0$, the following approximation applies:

$$P_j(x) \cong [1 - B(x) \text{PROFAC}_j] [1 - \exp(-H_{j,0})]. \quad (13)$$

In this case the relative risk, $RR(x)$, for episilencing tumor-suppressor-*miRNA* gene j is given by the following:

$$RR(x) = P_j(x) / [1 - \exp(-H_{j,0})] \cong 1 - B(x) \text{PROFAC}_j. \quad (14)$$

A similar relationship as used in Equation 14 has been used for deriving a benefit function for the prevention of lung cancer by residential radon exposure (Scott 2011a). The impacts of $B(x)$ and PROFAC_j on the shape of the dose-response curve for tumor-suppressor-*miRNA* gene episilencing will be investigated in detail in our future research. Where adaptive-response data are available, point estimates of $B(x)$ can be obtained (Scott 2011a). Our future research will also explore how benefit functions for effects such as tumor-suppressor gene activation, DNA repair induction, and anticancer immunity induction contribute to the benefit function for lung cancer suppression.

The present study has focused on the shape of the dose-response curve for the attributable risk, $\psi_j(x)$, for episilencing of tumor-suppressor-*miRNA* genes under circumstances where adaptive responses do not occur. While results presented for low ($P_j(x)$ applies) and moderate ($P_j(x)$ or $\psi_j(x)$ applies) doses are expected to be reliable, the results may not be reliable for high doses. For high doses, differential cell killing as well as reactivation of episilenced *miRNA* genes (possibly via a novel mechanism [Pruitt *et al.* 2006; Weber *et al.* 2007]) may be important and this will be addressed in future research.

DIFFERENTIAL EFFECTS OF LOW- AND HIGH-LET RADIATIONS ON *MiRNA* EPISILENCING

Research by Aypar *et al.* (2011) focused on epigenetic alterations after low and high-LET irradiations. GM1011 cells were irradiated with low-LET X-rays and high-LET iron (Fe) ions and evaluated for DNA damage, cell survival and chromosomal instability. *Analysis of DNA methylation showed predominantly hypomethylation.* The researchers demonstrated that *miRNA* expression levels can be altered after X-ray irradiation and that these *miRNA* are involved in chromatin remodeling and DNA methylation, as was the case in the studies of Tellez *et al.* (2011) using chemical carcinogens (MNU/BPDE combination) that in contrast elevated the DNA methylation levels. A higher incidence of epigenetic changes was observed after exposure to X-rays than for exposure to Fe ions, even though the Fe ions elicited more chromosomal damage and cell killing.

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The distinction was apparent for the miRNA analyses where only three miRNAs involved in two major pathways were altered after high-LET irradiation while six miRNAs involved in five major pathways were altered after low-LET irradiation. Based on these findings, d_j and α_j are expected to be LET-dependent. *PROFAC* and $B(x)$ may also be LET-dependent since adaptive responses are usually more likely after low doses of low-LET radiation than after low doses of high-LET radiation.

Possibly the differential occurrence of adaptive responses after low- and high-LET radiation relates to the different miRNA signaling pathways that are associated with the two radiation types. For radon exposure, both low and high-LET radiations are involved and a pronounced adaptive response (radiation hormesis) has been demonstrated for low-level radon, related to suppressing sporadic lung cancer in humans (Scott 2011a; Thompson 2011). Such results suggest that under circumstances of combined exposure to low doses of both low- and high-LET radiation, protective miRNA signaling pathways associated with low-LET radiation may predominate over possibly deleterious pathways associated with high-LET radiation.

CONCLUSIONS

The following conclusions are based on the STEP1 model applications presented:

- For exposure to carcinogens at the low end of the known carcinogenic dose range, episilencing of tumor-suppressor-*miRNA* genes appears much more likely causative for entering the neoplastic transformation pathway than mutation induction.
- When the stochastic biological effect of interest after moderate doses is caused by episilencing of any single *miRNA* gene among an at-risk set of *miRNA* genes, then the hazard function for the biological effect would be expected to be a linear function of the carcinogen dose, with the slope of the line increasing as the number of *miRNA* genes in the at-risk set increases. Thus, the magnitude of the slope may be a useful measure of the size of the at-risk set of *miRNA* genes.
- When the stochastic biological effect of interest after moderate doses is caused by the simultaneous episilencing of n tumor-suppressor-*miRNA* genes, then the hazard function for the biological effect would be expected to be proportional to the carcinogen dose x raised to the n th power (i.e., x^n). For MNU/BPDE-induced neoplastic transformation of HBEC, simultaneous episilencing of the three tumor-suppressor-*miRNA* genes (*miR-200*, *miR-200b*, and *miR-205*) may be causal for entering the pathway to neoplastic transformation but this needs additional investigation as is being done at our Institute.

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- Tumor-suppressor-*miRNA* gene activation after low doses of a carcinogen may be the result of an epigenetic response (i.e., adaptive response). In such cases, the fold-change dose-response curve for the *miRNA* expression may have a hormetic shape (increasing at low doses where gene activation occurs and decreasing at high doses where gene epigenetic silencing occurs).

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