

# Bystander signaling via oxidative metabolism

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**Abstract:** The radiation-induced bystander effect (RIBE) is the initiation of biological end points in cells (bystander cells) that are not directly traversed by an incident-radiation track, but are in close proximity to cells that are receiving the radiation. RIBE has been indicted of causing DNA damage via oxidative stress, besides causing direct damage, inducing tumorigenesis, producing micronuclei, and causing apoptosis. RIBE is regulated by signaling proteins that are either endogenous or secreted by cells as a means of communication between cells, and can activate intracellular or intercellular oxidative metabolism that can further trigger signaling pathways of inflammation. Bystander signals can pass through gap junctions in attached cell lines, while the suspended cell lines transmit these signals via hormones and soluble proteins. This review provides the background information on how reactive oxygen species (ROS) act as bystander signals. Although ROS have a very short half-life and have a nanometer-scale sphere of influence, the wide variety of ROS produced via various sources can exert a cumulative effect, not only in forming DNA adducts but also setting up signaling pathways of inflammation, apoptosis, cell-cycle arrest, aging, and even tumorigenesis. This review outlines the sources of the bystander effect linked to ROS in a cell, and provides methods of investigation for researchers who would like to pursue this field of science.

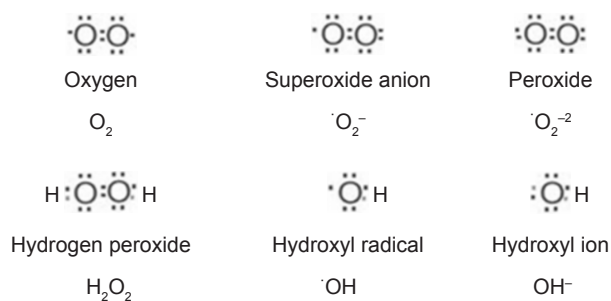
**Keywords:** radiation-induced bystander effect (RIBE), reactive oxygen species (ROS), oxidative stress, bystander signaling, tumorigenesis

## Introduction

Since the discovery of radiation-induced bystander effect (RIBE)-mediated mutations,<sup>1</sup> the associated gene expressions<sup>2</sup> and carcinogenesis<sup>3</sup> have been studied extensively. Several well-established signaling pathways of the RIBE have been established that include but are not limited to TGF- $\beta$ 1,<sup>4</sup> TNF $\alpha$ ,<sup>5</sup> IL8,<sup>6</sup> nitric oxide (NO),<sup>7,8</sup> COX2,<sup>9</sup> and carbon monoxide (CO).<sup>10</sup> On the basis of these factors, a unified model was proposed in 2008.<sup>11</sup> Recent research has shown that the regulation of the RIBE depends upon the secretion of certain important proteins that allow communication between cells, and could be as diverse as the reactive oxygen species (ROS) produced during oxidative metabolism that lead to stress.<sup>12</sup> Oxidative stress is mainly caused by increased levels of ROS in the environment of a cell. The generation of ROS is dependent on endogenous sources, such as by-products of cellular metabolism, or exogenous sources, such as exposure to ionizing radiation, ultraviolet radiation, or redox-cycling drugs, carcinogenic compounds, and anticancer alkylating agents.<sup>13</sup> Prolonged exposure to ROS leads to chronic inflammation, which contributes to tumor development and progression, and is hence recognized as a critical hallmark of cancer.<sup>14</sup> Some of the commonly occurring ROS species in radiation-targeted cells are shown in Figure 1.

Several biological abnormalities occur in both naïve and bystander cells. These abnormalities can be mutations and alterations in protein expression at transcription, posttranscription, translation, and even posttranslation levels.<sup>15-17</sup> These signaling

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**Figure 1** Commonly occurring reactive oxygen species in metabolically active cells.  
**Note:** Dots designate unpaired electrons.

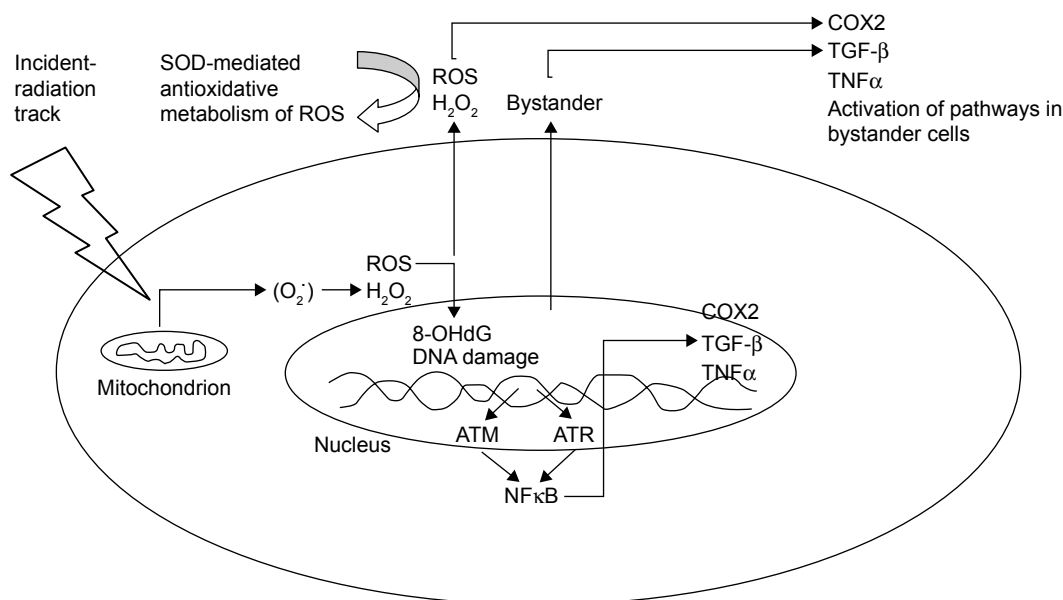
factors can affect bystander cells through the culture medium and/or the gap junction between directly communicating cells, but the source of this signal has been widely debated.<sup>8,18</sup> Evidence suggests that nuclear DNA could not be a direct target in the induction of the RIBE, but that the incident-radiation track must pass through the cytoplasm and membranous organelles before it reaches the nucleus, and hence any of the cytoplasmic organelles in the path of a radiation track could be the cause. It has been suggested that microbeam-targeted cytoplasmic irradiation can also induce bystander responses, where cell-membrane rafts may also be involved.<sup>19</sup> In addition, as key cell organelles in the cytoplasm, mitochondria have been shown to be sensors of the RIBE,<sup>20</sup> and the bystander response cannot be induced in cells without mitochondria.<sup>21–23</sup> The involvement of induced

DNA strand breaks or their repair pathways contributing to bystander mutagenesis has also been argued.<sup>24</sup> Considering the new advances in the RIBE, this review focuses on the signaling pathways that are involved in bystander signaling due to exogenous or endogenous sources of oxidative stress.

## Oxidative stress-related pathways of RIBE

In one of the earliest reported studies on the RIBE, it was shown that when glioblastoma cells were treated with helium ions, the frequency of micronuclei formation in the surrounding cells increased.<sup>1</sup> In normal cultures of fibroblast cells, it has been demonstrated that gap junctions, ROS, and TGF- $\beta$ 1 are involved in bystander signaling.<sup>2–4</sup> NO has been considered to contribute towards the initiation of damage to DNA in the medium.<sup>5,6</sup> Lyng et al<sup>122</sup> concluded that the RIBE led to the production of micronuclei and apoptosis<sup>7,8</sup> and that radiotherapy-induced stress can cause TGF- $\beta$ 1 release (Figure 2).

DNA damage-induced activation of ATM-p53 and ATM leads to the activation of NF $\kappa$ B-signaling pathways, followed by the stimulation of NF $\kappa$ B-dependent gene expression, including those for IL8, TNF $\alpha$ , COX2, iNOS, and the production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), ROS, and NO.<sup>23,25</sup> The stimulation of bystander cells due to the paracrine mechanism using cytokine or growth-factor interactions with the corresponding receptors results in an induction of cell-signaling



**Figure 2** Generation of ROS as a result of incident radiation affecting the mitochondrion.

**Notes:** ROS have been shown to form oxidative DNA adducts, such as 8-OHdG, which in turn can lead to base modifications and transversions, such as GC to TA. Any strand break would ensue the activation of a repair pathway via the repair kinases ATM or ATR.

**Abbreviations:** ROS, reactive oxygen species; OHdG, 8-hydroxydeoxyguanosine.

pathways and specific gene expression in bystander cells,<sup>9,23</sup> which in turn lead to the translocation of ROS and NO from directly irradiated to bystander cells through gap junctions with the secondary damaging effects on mitochondrial and genomic DNA in bystander cells.

## Genetic defects caused by radiation and ensuing signaling pathways

In similar experiments, it has been reported that after bombarding cells in vitro with radiation, DNA is damaged and genetic or chromosomal rearrangements occur. In mammalian cell lines, most genetic alterations occur in cells when they are exposed to a low frequency of  $\alpha$ -particles.<sup>11–14</sup> When radiation hits the target cell, because of its high-energy deposition (due to linear energy transfer), the chemical bonds in the target are cleaved and nucleotide bases damaged (Figure 3). Most lethal damage is due to the production of lesions, such as double-strand breaks, which occur because DNA in the nucleus becomes directly damaged. DNA damage can be repaired by the cells using DNA-repair machinery; however, if complete repair does not occur, there can be two possible outcomes: chromosomal abnormalities with depletion of genetic material that lead to cell-cycle arrest or the overwhelming extent of unrepaired damage could trigger apoptosis. The double-strand breaks thus caused can either be repaired efficiently via homologous recombination or with major defects via nonhomologous end joining and produce a bystander effect.<sup>11,17,19</sup>

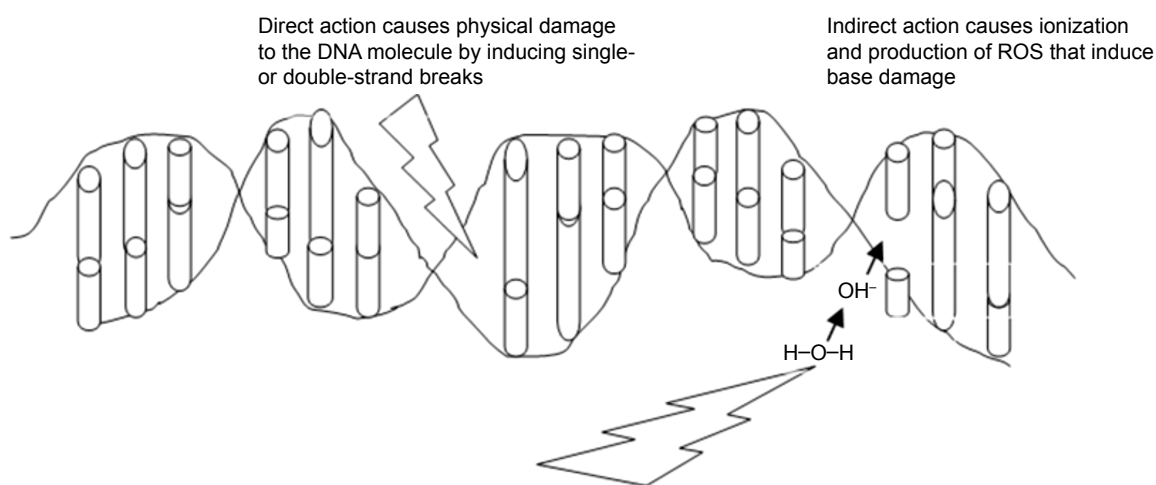
Accumulation of major damage over several generations can lead to tumorigenic progeny or ultimately to cell death.<sup>15</sup> The RIBE generally has two modes of action to proliferate bystander effects. For cells that are in direct contact with

each other, bystander signaling uses gap junctions. These tiny pores can transport small molecules between cells that are involved in signaling.<sup>16</sup> An important group of proteins called connexins combine with one another to form gap junctions. Such junctions can allow the transport of signaling molecules in the size range of 1–1.5 kDa. Important molecules that pass through these are small proteins, secondary messengers, and nucleotides. Attached cell lines, on the other hand, transfer signals through gap junctions, while the suspended cell lines transmit signals through released cytokines and other soluble molecules or even ROS.<sup>17,18</sup>

Microbeam low-dose irradiation has been used to target helium ions ( $^3\text{He}^{2+}$ ) to individual cells in a population of radioresistant glioma cells, either cultured alone or in coculture with primary human fibroblasts. The study indicated that even when a single cell within the glioma population had one  $^3\text{He}^{2+}$  ion pass through the cytoplasm, bystander responses were induced in the neighboring unirradiated glioma or fibroblasts. This resulted in a micronuclei yield increased by 36% for the glioma population and 78% for the bystander-fibroblast population.<sup>19</sup> Direct low linear energy-transfer radiation induces point mutations in mitochondrial DNA. Some of the genes that undergo deletion include *MTATP6*, *MTCO3*, *MTND3*, *MTND4L*, *MTND4*, *MTND5*, and five tRNAs. As a stress response, the number of mitochondria increases in cells traversed with low-dose radiation. Such mitochondria may proliferate more rapidly than in untargeted cells.<sup>20</sup>

## Oxidative stress and Keap1–Nrf2 stress-response pathway

Upon targeting with ionizing radiation or oxidative xenobiotics, cells are usually confronted with oxidative stress,



**Figure 3** Ionizing radiation affecting DNA, either physically to cause strand breaks or indirectly via ion production to cause base damage.

**Notes:** The ionized products can in turn also perpetuate DNA damage. However, due to the short half-life of these ions, the damage does not persist too long or spread too far.

**Abbreviation:** ROS, reactive oxygen species.

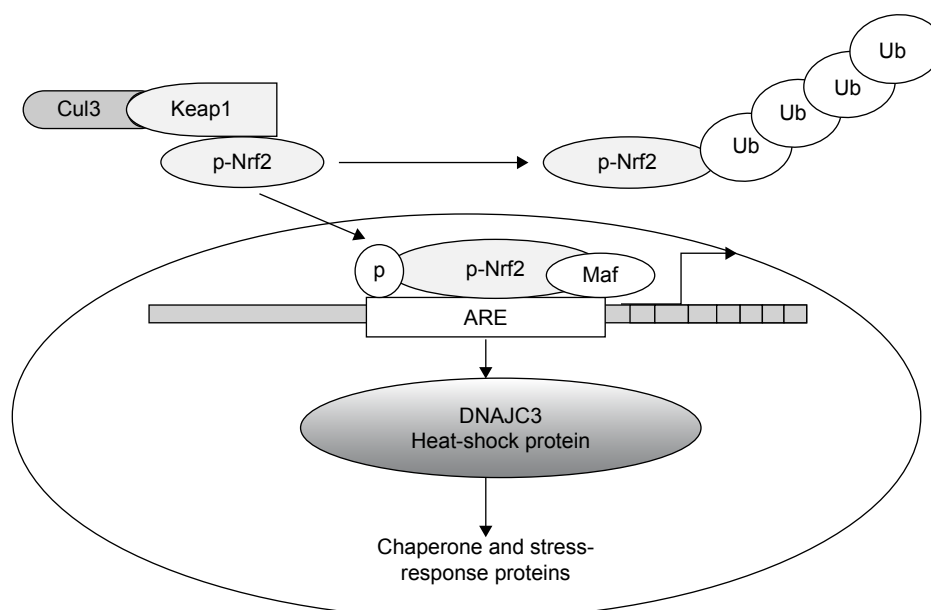
and hence they must quickly augment their antioxidant capacity to deal with increased ROS production and maintain homeostasis. Nrf2 is a transcription factor regulating the redox-homeostatic gene-regulatory network (Figure 4). The Nrf2-signaling pathway is activated under oxidative stress to increase the expression of a number of antioxidants and drug-detoxification phase II enzymes, such as UGTs, that can jointly work to restore redox homeostasis. Keap1, a cysteine-rich protein, while being anchored to actin in the cytosol, interacts with Nrf2 to work as an adaptor protein for the Cul3 ubiquitin-ligase complex. Under normal conditions, Keap1 promotes ubiquitination and eventual degradation of Nrf2. This is a very quick event, because Nrf2 has a relatively short half-life of 13–21 minutes.<sup>26,27</sup> The Keap1–Nrf2 pathway plays a pivotal role in providing protection against oxidative and xenobiotic stresses. The Nrf2 transcription factor activates the transcription of various cytoprotective genes that have been indicted in cancer and neurodegenerative diseases.<sup>28</sup> In fact, this pathway is now recognized as an important anticancer target.<sup>29</sup> While the Keap1–Nrf2 pathway enables the cell to counter oxidative and xenobiotic stresses, its disruption does quite the opposite, making the cell susceptible to carcinogens and chronic inflammation that eventually modify the phenotype of a cell from normal to tumorigenic.<sup>30,31</sup> The importance of Nrf2 has been highlighted in the gene regulation of several phase II drug-metabolism enzymes. Homeostatic regulatory control of the expression of phase II enzymes in a cell is Nrf2-dependent.<sup>32</sup>

At genetic and transcription levels, Nrf2 activation mediates bystander signaling via oxidative metabolism. The expression level of DNAJC3, sometimes known as protein kinase-inhibitor p58, was shown to increase by 1.6-fold in the untargeted liver of irradiated rats. DNAJC3 is known to be involved in the endoplasmic reticulum stress response. It is also a target of Nrf2, a critical transcription factor for mediating amplification of the mammalian defense system against various environmental stresses. The Nrf2 protein is a key regulator of response to oxidative stress. It acts as a transcription factor that regulates the expression and induction of a range of defensive genes encoding detoxifying enzymes and antioxidant proteins.<sup>36</sup>

### Bystander signaling at translation level

When simulated space radiation using <sup>48</sup>Ti ions at 50 cGy (1,100 MeV/nucleon) is targeted to the head of male Sprague Dawley rats, it can modify a number of proteins in the untargeted liver. Altered protein structure would eventually have modified function or cause complete loss of function. Although the list of cellular proteins that are modified or can possibly be modified is not exhaustive, one radiation-induced bystander-mutagenesis experiment reported 25 proteins to suffer that fate (Table 1).<sup>36</sup>

Some other ways in which signaling molecules could be modified are NO-dependent. NO-mediated posttranslational modification of macromolecules is a new area that we have



**Figure 4** The transcription level regulation of bystander signaling.

**Notes:** The transcription level regulation of bystander signaling via heatshock protein (DNAJC3) as it is activated by the inflammatory transcription factor called the nuclear transcription factor 2 (P-Nrf2). Maf is a small leucine zipper protein that combines with Nrf2 to bind the antioxidant response element (ARE), Kelch-like ECH-associated protein 1 (Keap1), Cul3 dependent E3 ligase (Cul3) regulates the cytosolic sequestration of Nrf2 via ubiquitination.

**Abbreviation:** ARE, antioxidant-response element.

**Table 1** Proteins modified in the head of a rat exposed to 50 cGy of radiation

Protein name	Accession no	Ratio (untargeted/control)
3-Ketoacyl-coA thiolase, mitochondrial	18426866	-2.37
$\alpha_1$ -antiproteinase	112889	-2.57
Ba 1-647	33086640	-2.17
Carbamoyl-phosphate synthetase I precursor	8393186	-2.57
Carboxyl esterase 3 precursor	140969642	3.67
Cathepsin D	42476045	-3.7
Choline dehydrogenase	1154950	-2.17
Fibrinogen $\gamma$ -chain	61098186	-1.57
Ganionic trypsin-I precursor	6981420	-2.17
Glycerate kinase	157821525	-2.57
Hydrolase B, serine esterase	3807109	3.67
Liver annexin-like protein	7108713	-2.57
Mitochondrial processing protease	397699	-2.57
Mitochondrial trifunctional protein, $\alpha$ -subunit precursor	148747393	-2.47
N-acetylneuraminase pyruvate lyase 2	157822207	-2.17
Precursor polypeptide (AA -18 to 547)	56899	3.67
Preproapolipoprotein AI	55747	-2.37
Protein disulfide-isomerase A3 precursor	8393322	-2.57
Protein kinase-inhibitor p58 precursor	11560030	1.57
Pyruvate carboxylase precursor	31543464	-1.57
Rieske Fe-S protein precursor	206681	-8.71
Serine protease inhibitor A3N	2507388	-3.07
Stress-70 protein, mitochondrial	116242506	-2.17
Sulfite oxidase	294639	1.57
T-kininogen I	60392582	-4.87

**Note:** Adapted with permission from Jain MR, Li M, Chen W, et al. In vivo space radiation-induced non-targeted responses: late effects on molecular signaling in mitochondria. *Curr Mol Pharmacol.* 2011;4(2):106-114.<sup>36</sup>

just now started to explore. Peroxynitrite, a strongly oxidizing intermediate of NO metabolism, has been shown to modify the structure of macromolecules to induce apoptosis in a p38-mediated manner.<sup>33</sup> Although the process is not yet fully known, it was clear that oxidative damage was not the primary cause,<sup>34</sup> but Akt and MLK/MAPK pathways are believed to be involved.<sup>35</sup> It is also logical to assume that the modified proteins (identified in Table 1) could have been due to the involvement of peroxynitrite.

## ROS-generating enzymes in cells

Several cellular organelles, such as mitochondria (as part of the electron-transport chain), the endoplasmic reticulum (especially during ER stress), and peroxisomes (in the metabolism of long-chain fatty acids),<sup>37</sup> regulate metabolic reactions that lead to ROS generation. Most of the enzymes involved in such metabolic reactions are either lipoxigenases, xanthine oxidases, cyclooxygenases, cytochrome P450 monooxygenases, NO synthases (NOSs), and NADPH oxidases.<sup>38</sup> Three types of such enzymes – lipoxigenases, cyclooxygenases and epoxygenases/monooxygenases (eg, cytochrome P450) – can metabolize arachidonic acid to biologically active eicosanoids. Various lipid peroxides

and bioactive lipids produced via this metabolism can affect cell proliferation, apoptosis, differentiation, and senescence, consequently leading to carcinogenesis.<sup>39,40</sup> An imbalance in the generation and removal of ROS can lead to a pathological condition associated with oxidative stress.<sup>41</sup> Prime candidates of oxidative stress are not only limited to the free radicals  $\cdot\text{OH}$ ,  $\text{RO}_2\cdot$ ,  $\text{NO}\cdot$  and  $\text{O}_2\cdot^-$ , but also include the nonradicals  $\text{HOCl}$ ,  $^1\text{O}_2$ ,  $\text{ONOO}^-$ ,  $\text{O}_3$ , and  $\text{H}_2\text{O}_2$ .<sup>42</sup>

## Lipoxygenase

Lipoxygenase (Lox)-catalyzed metabolism of arachidonic acid into various eicosanoids has been widely studied, and the enzyme expression varies throughout the phases of cancer initiation and progression, and hence regulates aspects of tumor development.<sup>43</sup> Lox comprises a family of nonheme iron dioxygenases that insert molecular oxygen into free and esterified polyunsaturated fatty acids and based on regional specificity may be classified as 5-, 8-, 12-, and 15-Lox.<sup>44</sup> The Lox enzymes metabolize arachidonic acid to the biologically active metabolite hydroperoxyeicosatetraenoic acids (HPETE), which can be reduced to hydroxyeicosatetraenoic acid (HETE). The products of Lox metabolism can either produce intermediary metabolites, such as HPETE, or they

can be catalyzed into secondary products, such as lipoxins, hepxilins, and HETE,<sup>45</sup> which can act as signaling molecules independently or give rise to ROS. The enzyme 15-Lox1 is expressed mainly in reticulocytes, eosinophils, epithelial cells of air passages, and macrophages of atherosclerotic lesions.<sup>46</sup> This enzyme has been shown to play a role in cell differentiation and maturation, inflammation, asthma, carcinogenesis, and atherogenesis.<sup>47</sup> While many investigations indicate that 5- and 12-Lox metabolites promote angiogenesis and carcinogenesis, in contrast 15-Lox may play an inhibitory role in tumor angiogenesis and thus may slow down carcinogenesis.<sup>48,49</sup> In a study that explored the contribution of photodynamic stress toward bystander signaling in WTK1 cells, lipid peroxidation was identified as playing a key role in the perpetuation of this signal.<sup>50</sup> The involvement of Lox enzymes in carcinogenesis and angiogenesis is a clear indication of the involvement of ROS, which could logically contribute to the overall bystander burden in an environment of oxidative stress.

## Cyclooxygenase

The role of Lox and COX is widely recognized in cancers.<sup>39,51,52</sup> Tumor-cell functions at primary and secondary sites are controlled by many different factors, including growth factors and their receptors, nuclear receptors, intercellular interactions, cell-matrix interactions, and various chemokines, as well as oxygenated metabolites of arachidonic acid.<sup>51,52</sup> In 1971, Vane first demonstrated that aspirin and indomethacin inhibited PG production by blocking the enzymatic activity of COX.<sup>53</sup> Since then, the direct correlation between COX and anti-inflammatory drugs, such as nonsteroidal anti-inflammatory drugs, has been explained. These drugs either covalently modify the enzyme or selectively inhibit (eg, COX2 inhibited by APHS, found in aspirin) by competing for the active site.<sup>54</sup> Aspirin blocks COX enzymatic activity via the acetylation of Ser530 in COX1 and Ser516 in COX2.<sup>55</sup> The COX1 and COX2 enzymes catalyze rate-limiting steps in the production of PGs and thromboxane from arachidonic acid. Both COX enzymes convert arachidonic acid to endoperoxide PGH<sub>2</sub>, and downstream-selective isomerases convert PGH<sub>2</sub> to prostacyclin, PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2α</sub>, or thromboxane A<sub>2</sub>.<sup>56</sup> COX2 expression has been shown to increase adenomas in mouse models of adenoma and implicated in human colorectal cancer, followed by the observation that COX2 also plays a role in colorectal metastasis into liver<sup>56</sup> and angiogenesis.<sup>57</sup> Also, the chemical inhibition of COX2 has demonstrated immense therapeutic significance for halting tumor growth and progression.<sup>58</sup> The enzymatic contribution

of Lox- or COX-mediated ROS toward bystander mutagenesis has not yet been investigated, and thus is a good direction to pursue.

## Cytochrome P450 enzymes

Cytochrome P450 enzymes (CYPs or P450s) are a large superfamily of monooxygenases present in all biological kingdoms.<sup>59</sup> Within the context of this review, they are of special interest, because their activity can cause oxidative stress not only via ROS production but also by the generation of reactive oxygenated metabolites (ROMs).<sup>62-64</sup> We summarize the current knowledge for both of these aspects.

P450s are B-type hemoproteins that are named after the unusual spectral properties of the complex between reduced CYP and carbon monoxide, which is characterized by a pronounced peak at 450 nm.<sup>60</sup> This spectral feature is caused by a cysteine-thiolate group that forms the fifth ligand of the heme iron. More than 200,000 distinct CYP genes have been identified to date<sup>61</sup> and P450 systems can be found throughout all life forms, although there are organisms (such as *Escherichia coli*) that lack them.<sup>62</sup> In general, CYPs are the terminal oxidase enzymes in short electron-transfer chains that encompass a CYP enzyme together with one or two electron-transfer proteins and constitute a P450 system.<sup>63</sup> P450 systems can metabolize a huge variety of small and large molecules, and are best known for catalyzing hydroxylations, but some of them can also perform *N*-, *O*-, and *S*-dealkylations, sulfoxidations, epoxidations, deaminations, and even other reaction types.<sup>64-66</sup>

In mammals, CYP content is highest in the liver, but they are also present in kidneys, small intestine, lungs, adrenal cortex, skin, brain, testes, placenta, and other tissues. Humans have 57 functional CYP genes, and the enzymes encoded by them are membrane-bound proteins that are either located on the cytoplasmic side of the endoplasmic reticulum or on the matrix side of the inner mitochondrial membrane. In addition, the nuclear envelope and plasma membranes contain low amounts of CYP.<sup>67,68</sup> Many human CYP systems are involved in the phase I metabolism of xenobiotics, while others have their predominant role in the biosynthesis of physiologically important compounds, such as steroids and fatty acids; in addition, around half a dozen members are still considered “orphans”, because their main function remains unknown.<sup>69</sup> Importantly, the two groups of drug-metabolizing CYPs on one hand and those CYPs that catalyze vital endogenous reactions on the other are not strictly separated from each other, eg, some steroid hydroxylases can also metabolize xenobiotics with steroidal structures.<sup>70-74</sup>

Despite the diversity of P450 chemistries, all cytochrome P450-enzyme reactions follow a common catalytic cycle, in which an iron(IV)-oxo species with an additional oxidizing equivalent delocalized over the porphyrin, and thiolate ligands (P450 compound I) are the principal intermediate.<sup>75</sup> According to current understanding, the individual steps in the P450 catalytic cycle are: 1) substrate binding, 2) first single-electron reduction, 3) oxygen binding, 4) second single-electron reduction, 5) protonation of the distal oxygen coordinating with iron, 6) formation of the reactive heme iron-oxygen species (compound I), 7) hydrogen-atom abstraction, 8) oxygen rebound with the radical intermediate, and 9) product release.<sup>76</sup> While the different steps of the cycle are believed to be very similar for most CYP enzymes, the rates of each step can differ widely between different CYPs and sometimes even for different reactions catalyzed by the same enzyme. For instance, the second electron transfer is the rate-limiting step for most bacterial P450 enzymes, which can reach turnover numbers of over 1,000/seconds. By comparison, eukaryotic steroid hydroxylases are typically much slower, and display turnover numbers in the range of 5–250/minute.

Importantly, the process of electron transfer during the activity of a P450 system may be either “coupled” or “leaky”: in a coupled P450 system, all electrons from NADPH are utilized in the biotransformation. However, if some electrons are transferred to other acceptors, then this process is referred to as “uncoupling” of electron transfer from biotransformation, as “leaky” electron transfer, or as “futile” oxidation. Uncoupling can occur during electron transfer from NADPH to the CYP enzyme and also during the P450-reaction cycle during the so-called shunt reactions that lead to production of water or ROS instead of substrate turnover.<sup>77</sup> These include the autoxidation shunt, which results in the formation of superoxide anion ( $O_2^-$ ) and the peroxide shunt that generates hydrogen peroxide ( $H_2O_2$ ).

Therefore, ROS are always generated during NADPH-dependent drug metabolism in liver microsomes, as well as in isolated hepatocytes, and CYP systems are a significant source of ROS in biological systems, especially in tissues like the liver, where P450 is present in high amounts.<sup>78</sup> It is important to remember that ROS can have important physiological effects on gene signaling and gene activation, so it is probably inappropriate to attribute only adverse effects to ROS generation by CYP systems.<sup>79</sup> Still, the toxicity of many reagents is at least partially due to the increased ROS production that occurs when they are metabolized by CYPs; some examples are acetaminophen, benzene,  $CCl_4$  and other halogenated

hydrocarbons, and nitrosamines. In addition, some xenobiotics are able to enhance their own metabolism by inducing the expression of CYPs that metabolize them, eg, polycyclic aromatic hydrocarbons and barbiturates, such as phenobarbital, increase CYP1A2 levels, while glucocorticoid drugs, such as dexamethasone, induce CYP3A4.<sup>80</sup>

ROS generation depends on several factors, including the specific form of CYP, entry of the second electron into the P450 cycle, and the presence and nature of the substrate.<sup>81–84</sup> For instance, one study determined a rank order for ROS-generating activity by microsomes from lymphoblasts expressing human CYPs to be CYP3A4 > CYP1A1 > CYP1A2 = CYP2B6.<sup>85</sup> CYP2E1 was repeatedly shown to be highly uncoupled,<sup>86,87</sup> and appears to be the most significant generator of ROS in liver. Its induction by chronic ethanol exposure is thought to contribute significantly to ethanol-induced oxidative stress and hepatotoxicity.<sup>80,88–91</sup> Significant evidence for this notion also comes from the observation that ROS production in E47 cells (ie, HepG2 cells that constitutively express recombinant human CYP2E1) is about 50% higher than in normal hepatocytes and associated with roughly 30% lower ATP levels, which is probably due to ROS damage to complex I of the respiratory chain.<sup>92</sup> ROS production by CYP2E1 also causes higher rates of microsomal lipid peroxidation within microsomes and liposomes, and this effect can be suppressed by inhibition of the enzyme’s activity with anti-CYP2E1 antibodies.<sup>93,94</sup>

In general, microsomal CYPs obtain the electrons necessary for their activity from cytochrome P450 reductase (CPR or POR), with cytochrome B5 sometimes acting as an allosteric factor to facilitate CPR–CYP interaction. After being reduced by cytochrome B5 reductase, cytochrome B5 may even act as an unusual donor for the second (but not the first) electron in the P450 cycle and has also been shown to be a source of ROS formation, albeit at lower levels than the CYPs themselves.<sup>90</sup> It is important to note that CPR protein levels in the ER membrane are typically much lower than those of all CYPs together, eg, the total CYP:CPR ratio is about 40:1 in rat hepatocytes.<sup>95</sup> Therefore, multiple P450 species compete for CPR interaction, which adds another dimension to the complexity of these processes.

As far as is known today, the seven human mitochondrial CYPs (CYP11A1, CYP11B1, CYP11B2, CYP24A1, CYP27A1, CYP27B1, and CYP27C1) are generally involved in adrenal steroidogenesis, bile-acid formation, and the metabolism of vitamins A and D.<sup>96–99</sup> Among these, CYP11A1, which catalyzes the conversion of cholesterol to pregnenolone (the rate-limiting step in adrenal steroid

biosynthesis), is the best-studied enzyme in terms of uncoupling and ROS production;<sup>100</sup> however, uncoupling in CYP11B enzymes might even be higher.<sup>101</sup> Many years ago, it was demonstrated in a seminal study that ROS are efficiently produced *in vitro* by CYP11A1 in the presence of NADPH and adrenodoxin reductase, but in the absence of its substrate cholesterol.<sup>102</sup> We later showed that transient overexpression of CYP11A1 decreased cell survival in some cancer cell lines but not in others; moreover, we showed that in those cases where loss of cell viability occurred, it depended on ROS formation.<sup>103</sup> In mammalian mitochondria, electrons are transferred from NADPH via adrenodoxin reductase (AdR or FdxR) to adrenodoxin (Adx or Fdx1), which in turn reduces mitochondrial CYP enzymes.<sup>63,104–106</sup> Therefore, as in the microsomal P450 system, the mitochondrial electron-transfer proteins are not specific for individual enzymes, but serve as electron donors for different CYPs in different tissues. AdR and Adx are expressed in all human tissues, and their highest levels of expression are observed in steroidogenic cells, especially in the adrenal cortex and ovarian corpus luteum.<sup>107,108</sup> However, with respect to their capability for ROS production, the properties of the two proteins differ remarkably: *in vitro* data show that while electrons can be transferred from NADPH via AdR and Adx to O<sub>2</sub>, AdR alone is not a major source of electron leakage in the presence of NADPH but without Adx.<sup>102,109,110</sup> Accordingly, AdR overexpression failed to exert an effect on cell proliferation in any cell lines tested, although it does sensitize cells to oxidative stress-induced apoptosis.<sup>103,111,112</sup> Therefore, in the absence of a mitochondrial CYP but in the presence of NADPH, Adx is the major source of ROS, so much so that it has been labeled an “electron gun” that is constantly being loaded and fired until electron supply via NADPH is exhausted.<sup>102</sup> Consistently with the results observed in reconstituted systems, transient overexpression of either human or bovine Adx led to reduced cell viability in all eleven tumor-derived and nontumorigenic cell lines tested.<sup>103</sup>

Formation of mitochondrial ROS is one of the major internal triggers for the initiation of apoptotic cell death.<sup>113</sup> An early step in this process is the release of cytochrome C from within the intermembrane space of the mitochondria, which then binds to Apaf1 to form apoptosomes. The apoptosomes bind to and activate caspase 9, which in turn activates downstream effector caspases, spreading a cascade of proteolytic activity that leads to breakdown of the mitochondrial membrane and eventually phagocytosis of the cell. Consistent with the process of apoptosis, we showed that transient overexpression of human Adx in HCT116 cells

caused a disruption of the mitochondrial transmembrane potential, to cytochrome C release from the mitochondria, and to increased caspase activity in the cell; by contrast, overexpression of an Apo mutant of adrenodoxin (C46S) that cannot bind the [2Fe-2S] cluster essential for electron transfer did not cause apoptosis.<sup>103</sup> Interestingly, recombinant expression of Adx does not cause apoptosis in the microbial model system *Schizosaccharomyces pombe*, in spite of a strong increase in ROS, which is indeed much higher than that observed in mammalian cells.<sup>114</sup> An explanation for this observation might well be connected to the very recent finding that *S. pombe*, in contrast to baker's yeast (*Saccharomyces cerevisiae*), does not age.<sup>115</sup>

As mentioned, CYP systems are not only a source of ROS but can also cause ROM-mediated oxidative stress to either the CYP enzymes themselves or the cells that express them. The transition intermediates formed in the CYP catalytic cycle are sometimes highly reactive and bind directly to the enzyme. Mechanism-based inhibition (also known as time-dependent inhibition) then occurs, and results in partial or total irreversible inactivation of the enzyme. Mechanism-based inactivation can potentiate the risk of drug–drug interactions and toxicity from coadministered drugs by increasing their steady-state levels. If the reactive species formed in the CYP are able to escape the active site and bind covalently to other proteins or nucleic acids, this can culminate in adverse drug reactions and toxicity.<sup>116</sup> Indeed, in the majority of cases, toxicity is caused by the actions of such reactive metabolites, rather than by the parent chemicals.<sup>116</sup> Generation of such ROMs is also a major reason for the failure of many drugs in pre- and postclinical trials.<sup>69</sup>

Although CYPs are quite well conserved, it is important to note that they are not identical, as polymorphic variants exist, sometimes at high frequencies. Even small differences in amino-acid sequence may have a significant impact on substrate affinity and turnover. This is exemplified by the considerable variation in the efficiency and amount of CYP2D6 produced between individuals, which cause drugs that are mainly metabolized by CYP2D6 to be eliminated by some individuals much more rapidly than by others. These variations give rise to four different drug-metabolizing phenotypes: poor metabolizers, which are characterized by a complete lack of enzyme activity due to two defective alleles; intermediate metabolizers, which are carriers of either one defective allele or two partially functional alleles; extensive metabolizers, which have two functional alleles; and ultra-rapid metabolizers, which have either multiple functional alleles or alleles that code for activated enzymes. Therefore, the dose of drugs that are mainly metabolized by CYP2D6



should be adjusted to take into account the speed at which they are metabolized by the individual patient. Increasing knowledge in this field has led to a transition from population-based dosing and prescriptions to patient individualization in both drug development and clinical practice, or in other words to personalized medicine.<sup>117,118</sup>

To complicate matters further, CYP-dependent drug metabolism is often followed by conjugation reactions catalyzed by phase II enzymes, such as UGTs or SULTs. In the context of safety testing, availability of all phase I and phase II metabolites is thus an essential prerequisite for the assessment of drug-caused side effects or toxicity.<sup>119,120</sup> Finally, differences in the activity of drug-metabolic enzymes in humans and animals are known to cause species differences in metabolism-related toxicity. An important example is the CYP-dependent metabolism of the breast cancer drug tamoxifen, which leads to the formation of desired active metabolites, such as 4-hydroxytamoxifen, but also to the undesired and DNA-reactive  $\alpha$ -hydroxytamoxifen. In human females, this latter metabolite is efficiently detoxified by UGTs, yielding a product that is rapidly eliminated from the body; rodent females lack sufficient UGT activity to derivatize all the  $\alpha$ -hydroxytamoxifen, and this reactive metabolite can then cause DNA damage and liver cancer.<sup>121</sup>

## Measurement of ROS production in bystander cells

The oxidative metabolism of  $\gamma$ -radiation-targeted cells can be investigated through the use of antioxidants, such as L-lactate and L-deprenyl, or functionally related drugs that can inhibit the collapse of mitochondrial membrane potential, resulting in the inhibition of cytotoxic effects on bystander cells when transferred from irradiated cultures. Chemical inhibition of the bystander signal with antioxidants signifies it to be ROS-based, while drugs that collapse mitochondrial membrane potential indicate the involvement of calcium-ion signaling or components of apoptotic signaling as possible candidates of bystander signal.<sup>122–124</sup>

## Other in vivo sources of free radicals

The redox state of a cell is generally linked to an iron/copper-redox couple and maintained within a strict physiological range.<sup>125</sup> This tight iron regulation ensures that there is no free intracellular iron; however, in vivo under stress, superoxide may sometimes release free iron. This phenomenon was demonstrated for the [4Fe-4S] cluster-containing enzymes of the dehydratase-lyase family.<sup>126</sup> The Fe(II) thus

released can take part in the Fenton reaction, producing highly reactive hydroxyl radical ( $\text{Fe[II]} + \text{H}_2\text{O}_2 \rightarrow \text{Fe[III]} + \cdot\text{OH} + \text{OH}^-$ ). Therefore, under stress conditions the  $\text{O}_2^-$  acts as an oxidant of the [4Fe-4S] cluster-containing enzymes and facilitates  $\cdot\text{OH}$  production from  $\text{H}_2\text{O}_2$  by providing Fe(II) for the Fenton reaction.<sup>127</sup> This hydroxyl radical has a half life of  $<1$  nanosecond, and thus in vivo it reacts very close to the source.<sup>128</sup> Production of  $\cdot\text{OH}$  close to the DNA can lead to its reaction with DNA bases or the deoxyribosyl backbone of DNA to produce damaged DNA strands. Another commonly occurring radical in cellular environments is the peroxy radical ( $\text{ROO}\cdot$ ). This radical is a high-energy species with a reduction potential varying between 0.77 and 1.44 V, depending on the functional group.<sup>129</sup> The role of peroxy radicals in DNA cleavage and disease is also well documented.<sup>130–132</sup>

## Free radicals as part of the immune defense mechanism

Oxygen-derived free radicals are purposely produced to neutralize invading viruses and bacteria. Macrophage- and neutrophil-mediated phagocytosis stimulates certain cellular mechanisms, including the “respiratory burst” that enhances oxygen uptake to produce potent oxidant bactericidal substances, such as hypochlorous acid and hydroxyl radicals. Simultaneously, NO, a gaseous radical produced by macrophages, reacts with superoxide to form peroxynitrite, to work as an additional bactericidal agent.<sup>133</sup>

While oxidants are continually being produced as by-products of normal metabolism, they are also rendered harmless by a range of antioxidant enzymes, such as glutathione peroxidase, glutathione reductase, catalase, and SOD, as well as nonenzymatic antioxidants, including thiol antioxidants, melatonin, coenzyme Q, and metal-chelating proteins, which are efficient enough to fight against excessive free radicals. Nutrient antioxidants, such as the most abundant fat-soluble vitamin E and the most abundant water-soluble vitamin C, also efficiently and effectively remove oxidants from the living system.<sup>134</sup>

This “two-faced” character of ROS has recently been characterized as a secondary messenger that induces and maintains the tumorigenic phenotype of cancer cells. DNA mutations are a critical step in cancer induction and progression, with enhanced levels of oxidative DNA lesions (8-OH-G) reported in various tumors.<sup>135</sup>

SODs are a class of metalloenzymes that are ubiquitously distributed among all oxygen-using organisms, and work by providing protection against the oxidative damage of high concentrations of superoxide radical anion ( $\text{O}_2^-$ ).<sup>136</sup>

The redox activity of SOD can be used<sup>137</sup> as the primary cellular defense against oxidative damage by simply reducing superoxide ( $O_2^{\cdot-}$ ) to oxygen ( $O_2$ ) and hydrogen peroxide ( $H_2O_2$ ). Overexpression of SOD has been demonstrated to have antiproliferative and antitumor effects *in vitro*. Oxidative stress as a source of ROS that leads to bystander signaling was shown to be inhibited with SOD treatment *in vitro*.<sup>138</sup> The ROS-induced mutation frequency in DNA that directly damaged and indirectly damaged (bystander) cells was able to be significantly reduced.

One of the most toxic and common species of ROS found *in vitro* is  $H_2O_2$ . It plays an important part in cell-cycle regulation and mitogenic stimulation. Measurement of  $H_2O_2$  *in vitro* can be facilitated by the use of homovanillic acid or Amplex red, which dimerizes upon oxidation by  $H_2O_2$  via horseradish-peroxidase catalysis. The monomer of both compounds is nonfluorescent, but becomes fluorescent upon dimerization with an emission wavelength of 425 nm and a peak excitation wavelength of 315 nm.<sup>139</sup> Colorimetric analysis of the sample using an enzyme-linked immunosorbent-assay reader can provide an optical density that can be quantified and plotted to measure ROS in the sample.

Superoxide radicals can be measured via an interaction between superoxide and another compound to produce quantifiable data. This can be achieved by the reduction of ferricytochrome C to ferrocyanochrome C. The transformation of citrate to isocitrate is catalyzed by the aconitase enzyme. This enzyme can be inactivated *in vitro* by superoxide through the oxidization of the Fe moiety from its cubane cluster. Subsequently, the concentrations of superoxide in culture can be estimated by the extent of enzyme inactivation. Activation of p53 and the Ras–Raf pathway was also implicated in oxidatively stressed conditions.<sup>139,140</sup> The ROS generated in cells usually have a very short half-life (nanosecond scale), and can travel only a short distance (micrometer scale) before being neutralized. Therefore, the readout obtained using certain techniques is not a very accurate measure, although new fluorescent probes are being developed to get a high-throughput analysis via live-cell imaging.<sup>149</sup>

## Reactive nitrogen species

Another oxidative species produced as a result of radiation targeting and hence usually studied in bystander signaling is NO. It is another commonly found reactive species, specifically referred to as reactive nitrogen species (RNS). RNS are a group of compounds with different properties and reactivity. Some RNS are highly reactive, and their interaction with target macromolecules can lead to permanent

modification of the target, suggesting that RNS might not be cell-signal transducers; however, our understanding of RNS as oxidizers of macromolecules has evolved over the years.<sup>141</sup> Nitrated proteins are removed by degradation<sup>142</sup> and other mechanisms that are not yet fully understood.<sup>143</sup> NO is produced from L-arginine by three main isoforms of NOS: epithelial (eNOS), neuronal (nNOS), and inducible (iNOS). The isoforms eNOS and nNOS are tightly regulated by calcium in a calmodulin-dependent manner.<sup>144</sup> On the other hand, the inducible isoform iNOS is present in hepatocytes, macrophages, and fibroblasts, and can produce large amounts of NO in response to inflammatory stimuli, such as ionizing radiation and oxidative xenobiotics, and provides host defense through its oxidative toxicity. A standard way to determine NO is to measure composition products, such as nitrate ( $NO_3$ ) and nitrite ( $NO_2$ ), colorimetrically. This reaction is catalyzed by nitrate reductase as  $NO_3$  is first reduced to  $NO_2$ .<sup>139,140</sup> iNOS is activated in response to a variety of cytokine and endotoxin signals that can lead to fast production of huge fluxes of NO. iNOS expression is also tightly regulated by MAPK and JNK–STAT pathways.<sup>145</sup>

## End points of bystander effect produced by targeted cells

Cell proliferation has been widely used as an end point of bystander signaling. Oxidative stress causes the proliferation process to slow down dramatically. This has been shown through several *in vitro* and *in vivo* experiments. When directly targeted (exposed to radiation directly), epithelial cells of rat liver were mixed with naïve bystander cells (not exposed to radiation directly) and incubated together for 24 hours. The naïve cells demonstrated a rate of cell proliferation that was higher by 14%–17% than the directly targeted cells.<sup>146</sup>

The bystander effect has been checked by using the membrane-localizing deuteroporphyrin (dimethyl ester) dye and separating the direct and bystander cells in a migration transwell chamber. The extranuclear localization of the photosensitizer used suggested that primary DNA damage is not the trigger for bystander response in naïve cells. The end points measured included elevated oxidative stress, DNA damage (micronucleus formation), mutagenesis, and decreased clonogenic survival. Furthermore, the antioxidant effect of vitamin E in targeted cells was shown to be responsible for preventing oxidative stress.<sup>50</sup>

A range of end points can be measured in direct and naïve bystander cells to quantify overall bystander effects, and the list below is only a few of them:

- genetic or epigenetic<sup>16</sup>
- small- or large-scale mutations or loss of heterozygosity<sup>17</sup>
- protein-signaling pathways<sup>11,15</sup>
- DNA damage-sensor proteins, such as  $\gamma$ -H<sub>2</sub>AX<sup>148</sup>
- proliferation and apoptosis in direct or naïve cells<sup>122,149</sup>
- sister-chromatid exchanges in direct or naïve cells<sup>1</sup>
- micronucleation in direct or naïve cells<sup>2,150</sup>
- neoplastic transformation in naïve cells.<sup>17</sup>

## Cell models used for RIBE

In many of the *in vitro* studies mentioned in this article, lymphoblasts were commonly used as cell models, primarily in conditioned-culture studies. This is an asset, because lymphoblasts are suspended cell lines and can be spun down easily without having to trypsinize the cells. Conditioned medium can then be decanted and applied to naïve cells for bystander analysis. Some cell lines used as models for the RIBE include TK6 (p53 wild), WTK1 (p53 mutant), and NH32 (p53 null). These cell lines also provide an added layer of analytical advantage, due to their p53 status.<sup>164</sup>

For bystander effects on human development, the use of stem cells is inevitable. Human stem cells are responsible for many important biological developmental processes, including growth and differentiation of embryos, environment maintenance, and aging. Regarding the RIBE in human stem cells, not much research has been conducted, but it was reported in 2010 that these cells are not prone to bystander signaling in comparison to other somatic cells in humans.<sup>151</sup> Fish cell lines have also been used for qualitative bystander-effect analysis. Different types of bystander effects produced in different target cell lines have been identified, but already-established fish cell lines do not generate death signals due to bystander effect.<sup>152</sup>

In 2009, the role of p53 was reported by Zhao et al.<sup>121</sup> Three liver cancer cell lines were used in this study: HepG2 (p53 wild type), PLC/PRF/5 (p53 mutated), and Hep3B (p53 null). In order to elucidate the RIBE, all of them were exposed to  $\gamma$ -radiation and then cultured with normal Chang hepatocytes having p53 wild type. Only HepG2 cells were able to initiate bystander signaling in surrounding normal Chang hepatocytes. When inhibitors of p53 and cytochrome C were used with HepG2 cells, the bystander effect was able to be downregulated. Radiation targeting in these cell lines activated cytochrome C only in HepG2 cells because of p53. Conclusively, it can be said that cytochrome C release is p53-dependent in the RIBE.<sup>153</sup>

A group has also investigated the production of bystander signaling in primary tissue culture under *in vitro* conditions.

Bladder samples were taken from mice and then treated with radiation, and irradiated tissue-conditioned medium taken from directly exposed cultures was applied to naïve tissue. The subsequent bystander signal produced was of high intensity compared to single cells, and so was the nuclear fragmentation that results in micronucleation. It was concluded that the extent of the bystander effect induced in 3D geometric tissue was higher when compared to single cells.<sup>154</sup>

## Chemical signals from targeted cells activate/stimulate various signaling pathways in naïve cells

It has been discovered that in bystander signaling between irradiated and unirradiated cells, cytokines play an important role.<sup>155,156</sup> A group of scientists have reported the inflammatory and proinflammatory action of cytokines by using the culture medium in which they treated human glioblastoma cells with different dosage of radiation. IL6 is an important cytokine with multiple functions, eg, in cancer, it causes malignancy and helps evasion of apoptosis.<sup>157,158</sup> IL8 levels have also been shown to increase in human gliomas, and it has an important role in tumor progression.<sup>159</sup> Generally, the immune system is involved in the production of different cytokines against disease. In one study, a heavy-ion beam was used to target T cells, in order to check the production of IL6 and TNF $\alpha$ . It was determined that cytokine generation decreased in the directly targeted cells after exposure to radiation, and might have been induced in the indirect cells through the bystander effect. In the cells that are directly exposed to radiation, the expressions of genes of various cytokines that are dependent on NF $\kappa$ B produced some other cytokines that are involved in autocrine and paracrine signals, which further initiated signaling processes involving the expression of NF $\kappa$ B in bystander cells. After radiation therapy, the changes produced in DNA because of heredity can be transmitted to many generations, and the naïve bystander cells have the same abnormalities in the genome as in the directly targeted cells.<sup>160</sup>

Genetic abnormalities and apoptosis are initiated because of radiation, which ultimately leads to DNA damage. When bone marrow in mice is exposed to radiation, it leads to cell death and DNA damage in bone marrow cells not directly targeted by radiation. Signaling in this scenario shows the involvement of various cytokines.<sup>161</sup>

Some bioinformatic tools have also been utilized to analyze gene modulation and signaling at early stages after the exposure of radiation in IMR90 fibroblasts. Real-time quantitative polymerase chain reaction and whole-human-genome

microarrays were the techniques used to authenticate an *in silico* analysis. For signaling studies specifically, semiquantitative image analysis and immunoblotting revealed the involvement of NF $\kappa$ B and Akt–GSK3 $\beta$  pathways.<sup>162</sup>

In 2013, Guo et al reported on the RIBE between germ cells and somatic cells in *Caenorhabditis elegans*. Radiation given to posterior pharynx bulbs and tails of *C. elegans* increased germ-cell death, elevated DNA damage, and introduced genomic inconsistencies in the F1 generation, which demonstrated a risk of tumorigenic phenotype in the next generation. RIBE-induced germ-cell apoptosis also showed a complicated collaboration among many signaling pathways in somatic and germ cells.<sup>163</sup>

## Conclusion

The RIBE can play a critical role in initiating secondary tumorigenesis via oxidative stress. The ROS produced as a result of radiation exposure can act as secondary messengers to cause bystander effects in naïve cells. Although the ROS have a very short half-life (nanoseconds) and travel only short distances (micrometers), their cumulative effect in cells directly targeted with radiation can trigger the activation of bystander-signaling pathways. Some sources of generation of ROS in a cell include direct ionization of molecules targeted with ionizing radiation or metabolic enzymes, such as COX, Lox, and monooxygenases. These ROS or ROM can set up intracellular and intercellular communication. Gap-junction-dependent signaling proteins in attached cell lines or soluble signaling proteins in suspended cell lines can communicate with neighboring cells via hormones and cytokines, which can activate several signaling pathways, including NF $\kappa$ B, TGF- $\beta$ 1, TNF $\alpha$ , and COX2, either in the directly targeted cells or naïve bystander cells. Another recently discovered stress-response pathway is mediated by Nrf2, which is a transcription factor regulating the redox-homeostatic gene-regulatory network. The Nrf2-signaling pathway is activated under oxidative stress to increase the expression of a number of antioxidant and drug-detoxification phase II enzymes, such as UGTs, which contribute to restoring redox homeostasis.

DNA damage, genetic changes, and double-strand breaks are the other consequences of radiation targeting. Because of radiation, the rate of division in bystander cells (indirect cells) is comparatively higher compared to exposed cells (direct cells). The antioxidative properties of vitamin E reduce oxidative stress. In developmental analyses, the germ-cell death rate shows that complex signaling is involved between somatic and germ cells. If somatic cells are under radiation exposure, there is a risk of cancer initiation and progression

in the next generation. Human stem cells are not responsive to bystander signaling, and untargeted effects are produced if only cytoplasm is exposed to radiation but not the nucleus. Therefore, the nucleus cannot be the primary cause of a DNA damage-induced bystander effect. Because of radiation, genetic changes introduced in DNA can be transmitted to the next generation if not repaired.

## Disclosure

The authors report no conflicts of interest in this work.

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