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Comprehensive Invited Review

Redox Regulation of Multidrug Resistance in Cancer Chemotherapy: Molecular Mechanisms and Therapeutic Opportunities

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Abstract

The development of multidrug resistance to cancer chemotherapy is a major obstacle to the effective treatment of human malignancies. It has been established that membrane proteins, notably multidrug resistance (MDR), multidrug resistance protein (MRP), and breast cancer resistance protein (BCRP) of the ATP binding cassette (ABC) transporter family encoding efflux pumps, play important roles in the development of multidrug resistance. Overexpression of these transporters has been observed frequently in many types of human malignancies and correlated with poor responses to chemotherapeutic agents. Evidence has accumulated showing that redox signals are activated in response to drug treatments that affect the expression and activity of these transporters by multiple mechanisms, including (a) conformational changes in the transporters, (b) regulation of the biosynthesis cofactors required for the transporter's function, (c) regulation of the expression of transporters at transcriptional, posttranscriptional, and epigenetic levels, and (d) amplification of the copy number of genes encoding these transporters. This review describes various specific factors and their relevant signaling pathways that are involved in the regulation. Finally, the roles of redox signaling in the maintenance and evolution of cancer stem cells and their implications in the development of intrinsic and acquired multidrug resistance in cancer chemotherapy are discussed. *Antioxid. Redox Signal*. 11, 99–133.

I. Introduction

The constant assault by environmental toxic substances over the evolutionary time has made it necessary for living organisms to develop mechanisms to avoid the deleterious effects of these cytotoxic compounds. Among the many important detoxification mechanisms, one that involves efflux pumps that actively eliminate toxic substances from inside the cells has been widely documented from bacteria to mammals. As many antitumor agents are derived from natural products, overexpression of these efflux pumps in cancer cells represents a major mechanism of drug resistance in cancer chemotherapy. What is intriguing is that most often, upregulation of a single efflux pump is associated with the development of pleiotropic resistance to a wide spectrum of structurally and functionally unrelated anticancer drugs. In mammalian cells, this multidrug-resistance phenotype was first observed ~30 years ago. When cultured cells were exposed to a single cytotoxic agent, the resulting drug-resistant variants exhibited collateral resistance to a broad spectrum of cytotoxic agents [for reviews, see (31, 95, 134)]. These cytotoxic agents shared little similarity in their chemical structures or modes of cytotoxicity. Molecular cloning and functional characterizations revealed that a single transmembrane protein named P-glycoprotein (Pgp) or multidrug transporter 1 (MDR1) was sufficient to give rise to the multidrug-resistance phenotype. It was later realized that MDR1/Pgp alone could not account for all the multidrug resistance in many independently established multidrug-resistant cells, which led to the discoveries of other multidrug resistance–related transporters, notably multidrug resistance–associated protein (MRP1) and breast cancer–resistance protein (BCRP). Amino acid sequence analyses revealed that all these multidrug-resistance proteins contain multiple transmembrane domains (TMDs) and intracellularly localized ATP binding cassette (ABC) (Fig. 1). These multiple TMDs form a pore whereby animal cells use the intracellularly localized ABC to hydrolyze ATP to provide an energy source to eliminate cytotoxic compounds outward and reduce intracellular drug content to a sublethal level. Thus, these multidrug transporters belong to the ABC-transporter superfamily.

The human genomic sequencing project revealed that humans have 49 ABC transporters (http://www.geme.ucl.ac.uk/nomenclature/genefamily/abc.html or http://www.nutrigene.4t.com/humanabc.htm). These 49 ABC transporters have been classified into seven groups (*i.e.*, ABCA1-ABCA12, ABCB1-ABCB11, ABCC1-ABCC13, ABCD1-ABCD4, ABCE1, ABCF1-ABCF3, and ABCG1-ABCG5). Their primary amino acid sequences define their chemical structures and their substrate specificities. Many of these ABC transporters are multidrug transporters of cancer chemotherapeutics.

Reactive oxygen species (ROS), including oxyl radicals such as superoxide anion [$O_2\cdot$], hydroxy radical [·OH], and hydrogen peroxide [H_2O_2], are generated endogenously by all aerobic cells as byproducts of many metabolic reactions. Mitochondria form the major powerhouse of ROS production; they are generated in association with the activity of the respiratory chain [*e.g.*, NADH dehydrogenase enzyme complexes (278)] in aerobic ATP production. In addition, two classic phagocytic ROS-generating enzymes use molecular oxygen as a substrate, including the multisubunit NADPH oxidase and its homologue NOX/Duox family (140), and myeloperoxidase (70) in various tissues in response to ex-

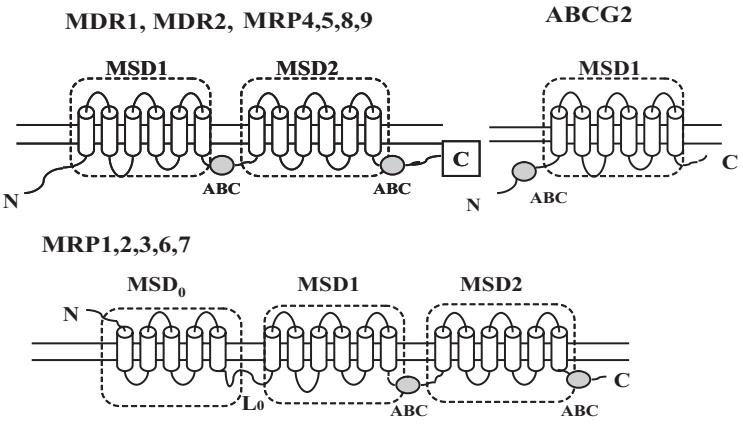

FIG. 1. Schematic diagrams showing the structure of various ABC transporters. MRP1, 2, 3, 6, and 7 each contain 17 transmembrane (TM) motifs distributed into three membrane-spanning domains (MSDs), whereas MDR1, MDR2, and MRP4, 5, 8, and 9 have 12 TMs in two MSDs, and ABCG2 has six TMs in one MSD. All these proteins are ABC transporters that containing intracellularly localized ATP-binding cassettes (ABCs).

tracellular influences (123). Other sources of ROS production include the cytochrome P450 (CYP450) system, which is involved mainly in removing or detoxifying toxic substances in the liver; xanthine oxidase, which catalyzes the oxidation of hypoxanthine to xanthine with the formation of H_2O_2; and nitric oxide synthase, which catalyzes a five-electron oxidation of a guanidino nitrogen of L-arginine in the formation of citrulline and nitric oxide.

Under normal physiologic conditions, a balance exists between oxidants and antioxidants: a reduction–oxidative (redox) homeostasis. ROS at submicromolar levels act as second messengers to stimulate cell proliferation, apoptosis, and gene expression. Endogenously elevated ROS are handled by antioxidants produced by a host of intracellular enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, and thioredoxin reductase (Trx) and the alpha class of glutathione S-transferase, which can decompose lipid hydroperoxides and hydrogen peroxide (237). Excessive levels of ROS produce oxidative stress that leads to a variety of diseases, including neurologic disorders, aging, and cancer. Moreover, oxidative stress can affect the efficacy of cancer treatments by multiple mechanisms, including chemosensitivity, apoptosis, angiogenesis, metastasis, inflammatory reaction, and chemosensitivity. In this review, the effects of redox signals on drug resistance, specifically on multidrug resistance encoded by a subset of ABC transporters, are presented.

II. Structure and Function of Multidrug-Resistance Transporter Protein Families

A. MDR/Pgp family

MDR belongs to the B group of the ABC transporter superfamily. Two Pgp-encoded *MDR* genes have been found in humans, *MDR1* (ABCB1) and *MDR2* (ABCB2). These membrane proteins contain two core membrane-spanning domains, MSD1 and MSD2, each of which consists of six transmembrane domains (TMDs) (Fig. 1). The two MSDs are separated by the first ABC. The second ABC is located at the C-terminus. Many review articles have described the structural and functional aspects of *MDR1*-encoding P-glycoprotein (Pgp) (31, 95, 133, 229). A brief background review relevant to the subjects of the present review follows.

The human *MDR1* gene is expressed in many normal tissues, including the liver, kidney, small intestine, colon, adrenal gland, and the blood–brain barrier, whereas *MDR2*/Pgp2 is expressed mainly in the liver and kidney. The endogenous substrates of Pgp1 remain to be determined, whereas Pgp2, which is expressed mainly in the liver canalicular compartment, functions as a phospholipid transporter. Only Pgp1 functions as an antitumor drug transporter for doxorubicin, vincristine, and taxanes, etoposide, teniposide, actinomycin D, *etc*. These substrates, although structurally dissimilar, are generally hydrophobic. It is believed that these agents enter the cells through passive diffusion due to a drug concentration gradient across the membrane and are eliminated against the concentration gradient by Pgp1 by using its intracellularly located nucleotide-binding domain (NBD) to hydrolyze ATP as an energy source. However, the molecular mechanism by which a single multispecific membrane protein can transport such a structurally diverse spectrum of antitumor agents has been a challenging topic since the completion of molecular cloning of *MDR* gene.

Because it has been unusually difficult to obtain the detailed molecular structure of drug-transporter proteins, owing to protein purification and crystallization of membrane proteins, the molecular basis of transport mechanisms remains largely unknown. Nonetheless, crystallographic information has become available from a bacterial homologue of multidrug transporter MsbA, providing instrumental information in elucidating the transport mechanism of Pgp (95). This information, together with studies using biochemical approaches (31), suggests that the initial event in Pgp-mediated drug transport is substrate binding, which leads to conformational changes that bring the two NBDs into close proximity to facilitate ATP binding. Nucleotide binding and subsequent ATP hydrolysis provide the energy needed to release the substrate outward through the multi-TMD–forming pore. Nonetheless, the detailed dynamic and vectorial processes involved remain largely unknown.

Mice have three *mdr* genes, two of which (*mdr1a* and *mdr1b*) are multidrug transporters, whereas the third (*mdr2*) has a function similar to that of human *MDR2*. Like those in the human counterparts, the endogenous substrates for *mdr1a* and *mdr1b* are not known. Mutant *mdr1*a(−/−) mice or compound *mdr1a*(−/−)*mdr1b*(−/−) mutant mice generated by knockout strategies are viable and fertile, suggesting that *mdr1a* and *mdr1b* are not essential for cell viability

(225, 226). However, these animals exhibit elevated sensitivity to the effects of cytotoxic agents. In this context, it is considered that animals use these efflux pumps to prevent accumulation of xenobiotics from various organs, thereby performing detoxification functions.

B. MRP/GS-X pump family

Nine members of the multidrug-resistance protein (MRP) family are found in the human genome, designated MRP1 through MRP9, corresponding to ABCC1 through ABCC6 and ABCC10 through ABCC12, respectively. Among the nine members, MRP4, MRP5, MRP8, and MRP9 have topologic configurations similar to those of MDR1 and *MDR2* (Fig. 1), whereas MRP1, 2, 3, 6, and 7 have an additional membrane-spanning domain, called MSD_0, located at the N-terminus. Topologically, MSD_0 is not essential for the catalytic function of MRP1, because deleting this domain did not compromise its substrate-transport activity (14). However, studies show that mutations at certain Cys residues within MSD_0 drastically reduce substrate-transport activities (153, 283), and a photoaffinity labeling study demonstrated that MSD_0 interacts with a photoreactive azido analogue of leukotriene C4 (LTC4) (121), suggesting that MSD_0 may not be entirely functionless. A recent study suggests that the N-terminal amino acid residues may function as a plug that controls the gating during drug transport mediated by the transporter (47). Many review articles regarding MRPs have already been published (31, 59, 131, 134). Only those that are most relevant to the current subject will be concisely discussed.

MRP1 was the first one in the family identified from a doxorubicin-resistant human cell line that did not overexpress MDR1/Pgp1 (52). The expression pattern of MRP1 is ubiquitous. Overexpression of MRP1 confers resistance to a spectrum of antitumor agents that is similar, but not identical, to that of Pgp1. Taxanes are good substrates for *MDR* but are poor substrates for MRP1. MRP2 has a substrate selectivity similar to that of MRP1, but the tissue-expression profile is quite different from that of MRP1. MRP2 expression is restricted to the liver and kidney. These tissues contain polarized cells. MRP2 is localized on the apical surface, predominantly in the canalicular membrane of hepatocytes. Its major physiologic function is to mediate ATP-dependent transport of glutathione (GSH) and glucuronate conjugate metabolites into the bile canaliculus in the livers. Both MRP1- and MRP2-mediated efflux require cofactors, glutathione GSH, glucuronic acid, or sulfate. MRP1 transports GSH conjugates such as LTC4, prostaglandin A2-SG, glucuronide conjugates such as 17β-estradiol-17-δ-glucuronide, and sulfate conjugates such as estron 3-sulfate sulfatolithocholate. Mice lacking *mrp1* are viable and fertile but have a defect in inflammatory response to the mediator LTC4, which is an endogenous substrate of *mrp1* (274). Animals with impaired *mrp2* expression showed defects in hepatobiliary extrusion of bilirubin glucuronide and developed the Dubin-Johnson syndrome (120).

The structural organization of MRP3 is similar to that of MRP1 and MRP2, but the substrate specificity of MRP3 is quite different from those of MRP1 and MRP2. MRP3-mediated transport does not require intracellular GSH. Moreover, in the liver, MRP2 is expressed in the canalicular compartment, whereas MRP3 is expressed in the basolateral membrane. MRP3 may be the major transporter involved in the basolateral transport of organic anions in the liver, because animals deficient in *mrp3* have defective transport of morphine-3-glucuronide from the liver into the bloodstream (289). Etoposide appears to be transported by MRP3 in unmodified form (288), whereas vincristine and doxorubicin, which are transported by MRP1 and MRP2 (but require no glutathionation with the drugs), are not transported by MRP3.

MRP4 and MRP5 contain 12 TMDs rather than 17 TMDs, making them structurally more like MDR1 than like MRP1. Cyclic nucleotides and their analogues are substrates of MRP4 (228) and MRP5 (206, 221) but not of MRP1, MRP2, or MRP3. MRP4 is expressed in the basolateral membrane of the choroid plexus epithelium and in the brain capillary endothelium. In *mrp4*(−/−) animals, the movement of topotecan from the blood into the brain tissue and cerebrospinal fluid is effectively limited, resulting in the accumulation of topotecan in the brain (145). These results suggest that MRP4 transports not only cyclic nucleotides but also antitumor agents and that MRP4 may have therapeutic value that targets the central nervous system.

MRP6 and MRP7 share structural similarities with MRP1 and MRP2 topologically. Like MRP2, MRP6 is expressed primarily in the liver and kidney. Expression of the MRP7 gene is ubiquitous but is high in the pancreas in the adult and fetal tissues. Increased MRP7 expression was detected in doxorubicin-treated MCF7 cells, suggesting that it is involved in resistance to antitumor agents. It has been demonstrated that MRP6 is capable of transporting GSH-conjugates such as LTC4 and DNP-GSH but not glucuronide conjugates such as E217βG, suggesting that MRP6 is a GS-X pump (131). The roles of GSH in the function of these transporters have not been as well characterized as those of other MRP transporters. Animals with ablated *mrp6* by knockout technology develop mineralization in connective skin, resembling the pseudoxanthoma elasticum syndrome (126). However, the molecular basis for the role of MRP6 in this disease is not clear.

MRP8 and *MRP9* genes are tandemly located in a tail-to-head orientation on human chromosome 16q12.1. The predicted amino acid sequences of both gene products show a high degree of similarity with MRP4 and MRP5, suggesting that the substrate specificities would be related to those of MRP4 and MRP5. This indeed has been the case. Ectopic expression of MRP8 in mammalian cells enhances cellular efflux of cyclic nucleotides and confers resistance to certain anticancer and antiviral nucleotide analogues (84). Interestingly, a single-nucleotide polymorphism in MRP8 (G538A) is associated with the dry-earwax phenotype in humans, and GA and GG, with the wet type (286) and colostrum and cerumen section (177). Recently, full-length MRP9 has been cloned. Expression of MRP9 was not detected in many human tissues except in the midpiece of sperm (192). The function of MRP9 and the identity of its substrate remain to be investigated.

C. BCRP/ABCG2

Human BCRP, also called mitoxantrone-resistance protein (MXR), belongs to ABCG2. It was first cloned from the dox-

orubicin-resistant MCF-7 breast cancer cell line in the presence of a Pgp inhibitor (5, 65, 178). This transporter encodes only 655 amino acids, about one-half of the size of MDR1 or MRP transporter (Fig. 1). It is thus likely that two half-molecules form a homodimer to function as a drug transporter. BCRP transports a diverse array of substrates including mitoxantrone, topotecan, doxorubicin, and SN-38, which overlap those of Pgp1 and MRP1 (20, 130, 271). It has been demonstrated that porphyrin and porphyrin-like compounds are natural substrates of BCRP (115). Imatinib (Gleevec) (100) and gefitinib (Iressa) (193), two important tyrosine kinase inhibitors developed for the treatment of chronic myeloid leukemia (CML), bearing p210Bcr-Abl abnormality, are also inhibitors of BCRP-mediated transport activities. BCRP apparently does not require GSH cofactor for transport. Like *mdr1a*(−/−), *mdr1b*(−/−), and *mrp*(−/−) mice, *bcrp*(−/−) animals are fertile with no apparent phenotypic alterations as compared with those in the wild-type animals, but are extremely sensitive to the pheophorbide a, a phototoxic porphyrin catabolite of chlorophyll (115). Furthermore, *bcrp*(−/−) animals also show increased fatal accumulation of topotecan (115). Because BCRP is expressed mainly in the placenta, it is suggested that BCRP may control the penetration of drugs from the maternal plasma into the fetus.

D. Non-ABC multidrug-resistance proteins

Another GSH-conjugate transporter, Ral-binding protein 1 (RLIP76/RALBP1), is a Ral-regulated protein that belongs to Rho family GTPases. Membrane-associated RLIP76 functions as a multispecific efflux pump for antitumor agents in vinca alkaloids and anthracyclines (9). RLIP76 appears to be a stress–response protein that provides protection from oxidative stress induced by these compounds.

Many reports have described the association of lung resistance–related protein (LRP) with non–Pgp-mediated multidrug-resistant phenotypes in human cancer cell lines. LRP is related to major vault protein (MVP), which consists of multiple subunits in a hollow barrel-like structure that functions in nucleo-cytoplasmic transport (224, 240). It has been proposed that MVP/LRP may act by transporting antitumor agents away from their subcellular targets, and supporting results have been published [for review, see ref. (183)]. However, it was reported that embryonic stem cells and bone marrow cells derived from murine *MVP/LRP*-knockout animals showed no difference in sensitivity to a wide spectrum of cytotoxic agents (182).

III. Redox Regulation of Multidrug-Resistance Transporters: Conformational Changes of the Transporters

The conformation of multiple membrane-spanning proteins is maintained by multiple parameters, including hydrogen bonds, hydrophobic effects, ion pairs, charge interactions, weak Van der Waal force, and intra- and intermolecular cysteine linkages. Several ABC transporters contain redox-sensitive amino acid residues such as cysteine (Cys). These Cys residues are subject to intramolecular disulfide formation, which is required for the maintenance of proper protein folding for their transporter's function. The eukaryotic machinery for controlling protein folding involving disulfide bond formation is carried out mainly by protein disulfide isomerase (PDI), which occurs as both oxidized and reduced forms (Fig. 2). Oxidized PDI acts as a disulfide donor in the oxidative protein folding of reduced protein. After the oxidation of a nascent protein, PDI is in the reduced state, which is reoxidized by a cascade consisting of endoplasmic reticulum oxidoreductin 1 (Ero1), its cofactor flavin adenine dinucleotide (FAD), and O_2. The consumption of O_2 as the terminal electron acceptor leads to oxidative stress through the production of ROS, which upregulate the production of GSH by upregulation of the expression of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme for the biosynthesis of GSH (see later). ROS also oxidizes GSH to GSSG, which is a substrate of MRP1-mediated transport (103).

The ratio between GSH and GSSG in cytosol is ~100:1, but in the endoplasmic reticulum (ER), it is ~3:1 (103). The maintenance of a sufficiently oxidizing environment in the ER is to compensate for disulfide formation. However, it is also known that Ero1 and PDI can drive the oxidation of protein folding, even in the reduced environment in which GSH is abundant. It is likely that Ero1- and PDI-mediated oxidative protein folding may gradually tip the equilibrium in favor of the formation of GSSG, whereas GSH-mediated reductive processes may feed back in maintenance of redox equilibrium. This dynamic redox homeostatic-regulation mechanism controlled by the coupled Ero1-PDI and GSH-GSSH systems may explain how ER supports rapid disulfide formation while it reserves the capacity to correct improper protein folding (262) (Fig. 2).

PDI contains two thioredoxin-like domains, each of which contains an active-site CXXC. Mutation in one of the active sites is sufficient to effect the folding of its target protein (98). Likewise, Ero1 has five known disulfide bonds, and two of them (Cys100-Cys105 and Cys 352-Cys355) are essential for the enzymatic activity *in vivo*. However, mutations in the noncatalytic cysteine pairs (Cys90-Cys-349 and Cys150-Cys295) affect Ero1 activity in a redox-dependent manner. When these cysteines are in reduced form, Ero1 is catalytically active; when they are in the oxidized form, Ero1 is inactive. These results demonstrate the importance of a redox environment in the regulation of the protein conformation of Ero1, which in turn regulates the global protein-folding machinery (236).

In addition to its function as a redox catalyst, PDI also functions as a chaperone by retaining proteins in the ER, thereby circumventing their protein degradation by the ubiquitin-proteasome pathway (83). Deregulation of these control mechanisms leads to the accumulation of unfolded protein in the ER; as a consequence, cells activate the intracellular unfolded protein–signaling pathway that triggers upregulation of a whole host of ER-stress proteins to overcome the stress. Perturbation of the capacity of this regulation, leading to excess production of disulfide bonds or misfolded mutant proteins, would ultimately lead to cell death.

The BCRP (ABCG2) protein contains 11 Cys residues. Three of them (Cys592, Cys 603, and Cys608) are located in the extracellular face between TM5 and TM6 (93, 117, 270). Cys592 and Cys608 are important for protein stability by forming intramolecular disulfide bonds. Mutations at these two Cys residues disrupt intramolecular disulfide linkage, resulting in protein misfolding and protein degradation, and

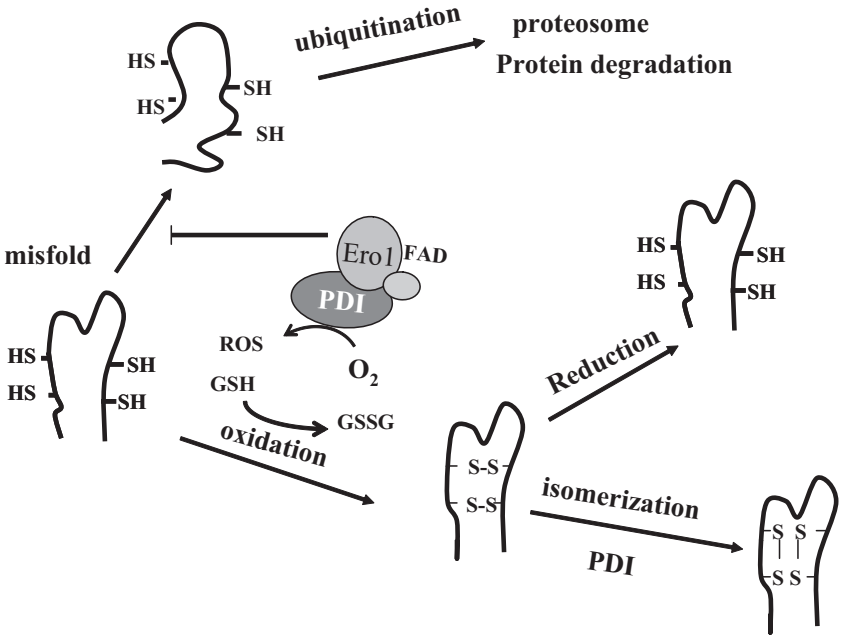

FIG. 2. Schematic model showing the regulation mechanism of oxidative protein folding in ER. Formation of disulfide bonds in the ER is driven by the Ero1, FAD, and PDI cascades. The reaction uses molecular O_2 as an electron acceptor that leads to the generation of ROS. PDI then carries out an isomerization reaction to produce proper protein folding. Reduced GSH may participate in the reduction of disulfide, resulting in the production of oxidized GSSG. Misfolded protein may be targeted to proteasome for protein degradation through ubiquitination. PDI and Ero1 are by themselves regulated by redox homeostasis. Formation of proper disulfide linkage by PDI leads to protein stabilization.

thereby increasing drug sensitivity because of the accumulation of the drugs (93, 117, 270).

Cys603 is involved in intermolecular disulfide bridge formation, resulting in dimerization of ABCG2. Mutation at this amino acid residue prevents the formation of homodimerization (270). Dimerization apparently does not require any amino acids other than those between TM5 and TM6. A recent study demonstrated that expressing a truncated BCRP consisting of TM5-loop-TM6 alone in cultured cells can oligomerize up to the dodecamer (93, 277). However, functional analyses revealed that mutation at Cys603 does not alter the transport activity of SN-38 and mitoxantrone by BCRP, although it has been generally perceived that monomeric ABCG2 represents a half-molecule of a functional ABC transporter. Recent study demonstrated that Cys284, Cys374, and Cys438 are potential sites for intramolecular disulfide bond formation and are required for the function of ABCG2 (158).

Intermolecular interactions involving critical Cys residues also occur in MRP family. As alluded to in Fig. 1, MRP1-3, 6, and 7 contain one extra MSD_0 at the N-terminus of the molecule. MSD_0 has been identified to be important for the dimerization of MRP1 (284). Dimerization of MRP1 is disrupted in the presence of dithiothreitol, suggesting that formation of disulfide linkage is mediated through the Cys residues. Yang *et al*. (283) investigated the roles of the two Cys residues (Cys7 and Cys32) located within the first 33 amino acids from the N-terminus of MRP1. These Cys residues are conserved among MRP1, 2, 3, 6, and 7 members. These investigators demonstrated that mutation at Cys7 drastically reduced LTC4 transport activities, whereas mutation at Cys32 did not (284). Mutations at Cys7 caused conformational changes and prevented dimerization in truncated MRP1 in co-transfection assay (283). It is, therefore, likely that this Cys residue may be involved in the dimerization of MRP1. As for Pgp1, whether functional Pgp requires the formation of the homodimer *in vivo* remains controversial (32). Currently, >15 PDI-like proteins are known in the family (67). It is not known which PDI-like protein is involved in the maintenance of protein foldings for these ABC transporters.

IV. Redox Regulation of Multidrug-Resistance Transporter Activity

Several members in the MRP family require GSH for the transport activities. GSH is the most abundant physiologic antioxidant, underscoring the roles of redox regulation of multidrug resistance mediated by this group of ABC transporters. The first identified endogenous substrate of MRP1 was LTC4 (113, 148, 184), which is a GSH conjugate immunomediator, consistent with the idea that MRP1 functions as a GS-X pump (108) (Fig. 3). The role of GSH in MRP1-mediated drug sensitivity in cultured cells was demonstrated: drug resistance in MRP1-overproducing cells could be par-

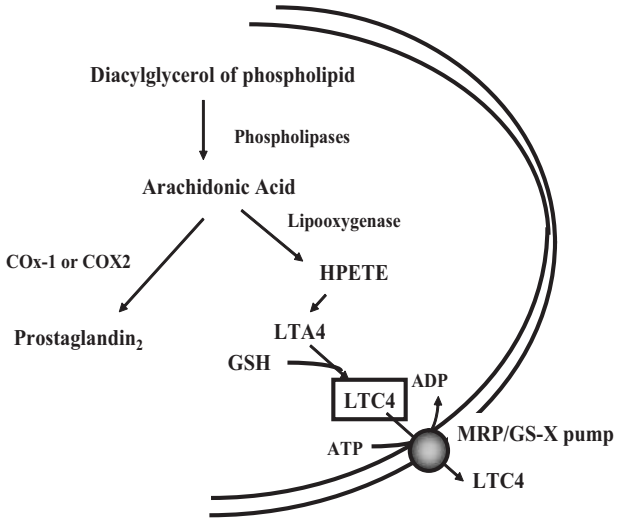

FIG. 3. Leukotriene biosynthesis and transport by the MRP1/GS-X pump. Leukotriene C4 (LTC4) is a GSH conjugate of LTA4, which is a metabolite of arachidonic acid by lipoxygenase through the intermediate hydroperoxyeicosatetraenoic acid (HPETE). Arachidonic acid is also metabolized by cyclooxygenases 1 and 2 (COX1, COX2) to prostaglandins.

tially reversed by exposing the cells to buthionine sulfoximine (BSO), an inhibitor of GSH synthesis. The effect of BSO on drug resistance was associated with decreased GSH levels and increased intracellular accumulation of daunorubicin owing to inhibition of the enhanced drug efflux. Increased GSH levels in BSO-treated cells by exposing cells in GSH ethyl ester restored the accumulation deficit of doxorubicin (268), demonstrating the critical role of GSH in the MRP1-mediated transport activity.

MRP1 also transports GSSG, the oxidized form of GSH, with relatively higher affinity than does GSH. Thus, MRP1 can be considered a regulator of GSH–GSSG homeostasis and its associated redox maintenance. Several lines of evidence suggest that the initial event in GSH-dependent MRP1-mediated drug transport involves the interaction between GSH and MRP1. GSH is a tripeptide consisting of Glu-Cys-Gly. Lesie *et al*. (151) investigated the GSH-binding domain of MRP1 by using a variety of amino acid substitutions in the tripeptide and concluded that, although other factors are contributory to the overall interactions, the molecular volume of the tripeptide is important for supporting MRP1 transport activity. Substituting the γ-Glu residues with Gly, β-Asp, and α-Glu resulted in the complete loss of transport stimulation of MRP-mediated transport using estrone 3-sulfate as a substrate; whereas substituting the internal Cys residue had no effect. These observations suggest a stereospecificity of GSH in supporting the MRP transporter. It has been reported that several short-chain GSH derivatives can stimulate MRP1-mediated vincristine uptake in a membrane vesical transport assay, suggesting that change in redox state and glutathionation of substrate is not involved (162).

By using the radioactively labeled photoactivatable GSH derivative, azidophenacyl-^{35}S-labeled GSH, which can functionally substitute for GSH, in an MRP1-mediated substrate (estrone 3-sulfate) transport assay, Qian *et al*. (203) found that the GSH directly interacts with two sites located at the N- and C-terminal halves of MRP1. This raises possibilities as to whether GSH functions as a co-transporter or a co-factor that stimulates the transport of the others. The co-transporter mechanism is based on the findings that transport of substrates requires GSH and that substrates can also increase the transport of GSH. Support of the co-transporter model can be found in that the MRP1-mediated transport of vincristine (162) and daunorubicin (DNR) (162, 210) in an isolated membrane vesicle supplemented the physical concentration of GSH. Moreover, the MRP1-mediated efflux of daunorubicin parallels that of GSH with a 1:1 stoichiometry when drug transport is saturated (219), supporting the earlier findings with respect to GSH and vincristine transports (147, 162). However, such reciprocal stimulation of transport was not found in the transport of nitrosamine metabolite, NNAL-*O*-glucuronide (152). Likewise, conjugated estrogen, estrone 3-sulfate, could be efficiently transported only in the presence of GSH, but no reciprocal increase was found in MRP1-mediated GSH transport (166). The co-transport model is even more complicated when inhibitors to MRP1-mediated efflux were used. Salerno *et al*. (220) reported that verapamil inhibits MRP1-mediated efflux of DNR, whereas GSH efflux is increased. MK571 inhibits the efflux of both DNR and GSH. These observations led these investigators to propose a model in which two interactive modules, one for the substrate and the other for GSH, worked in concert for the MRP-mediated transport.

One possible model proposed for the co-factor mechanism of GSH in MRP1-mediated drug transport is that the allosteric effect induced by GSH may lead to conformational changes in MRP1, rendering it more accessible to substrate binding. Induction of MRP1 conformational changes by GSH binding is supported by the results of a study using a limited tryptic-digestion approach (209). The investigators observed that GSH inhibited the tryptic digestion of the Lo region, which links MSD_0 and MSD1 of MRP1 in purified membrane vesicle (Fig. 1). By using reconstituted lipid vesicles containing an inside-out MRP1 configuration, Manciu *et al*. (166) showed that GSH binding induces a conformational change in MRP1 that affects the structural organization of the cytosolic domains, resulting in enhanced ATP binding or hydrolysis or both. Despite the intensive investigations, however, the detailed mechanisms underlying how the conformational changes led to the enhancement of MRP1-mediated drug transport remain to be learned. Very little information is available regarding the structural requirements in the substrates that are required for GSH to function as a co-factor for MRP1 transport (53). Moreover, it is unknown why some members of the MRP family require GSH as a co-factor, whereas others do not. All these issues require further investigations.

V. Redox Regulation of Multidrug-Resistance Transporter Gene Expression

A. MDR/Pgp family

Because the level of *MDR1* gene expression is frequently upregulated in human malignancies, understanding the upregulation mechanisms is important for designing strategies to circumvent the development of multidrug resistance in cancer chemotherapy. Nevertheless, most of our understanding of *MDR1* regulation mechanisms is from cultured cell studies. Multiple layers of regulation of *MDR1* gene expression have been demonstrated, including transcriptional, posttranslational, translational, and epigenetic levels. Moreover, the acquisition of multidrug resistance can be caused by gene amplification. These various layers of gene expression–regulation mechanisms are discussed.

1. Transcriptional regulation. Like many other RNA polymerase II–transcribed genes, *MDR1* promoter contains many transcription factor–binding sites (*e.g*., GC-boxes, CCAAT-, CAGGAACA-, and GCGTGGGCT- elements, which interact with Sp1, NF-Y, ets-1, and Egr transcription factors, respectively). *MDR1* promoter (and most of the MRP family members as well) lacks the TATA box. These transcriptional factors contribute to the bulk of the basal transcriptional regulation. Moreover, MDR1 expression can be induced by various stress conditions, including tumor promoter, UV and ionizing radiation, carcinogens, hypoxia, and chemotherapeutic agents. These are mostly stress-inducing agents that produce redox imbalance. Thus, various redox-induced signalings play important roles in transcriptional regulation of MDR1 expression. Here are some examples:

Carcinogens: Early investigations of the effects of carcinogens on *mdr* gene expression were focused on hepatocellular carcinoma (HCC) in rodents. HCC in the rats or mice can be induced by many different inducers (133). Teeter *et al*. (254) reported that the expression of the murine *mdr1a*, a paralogue

of the human *MDR1*, was overexpressed in four different liver-tumor models, including chemical induction with diethylnitrosamine (DEN), dimethylhydrazine, transgenic mice bearing hepatitis B viral (HBV) large-envelope polypeptide, and even spontaneous tumors. Interestingly, in rats, upregulation of *mdr1b* (another human *MDR1* paralogue) was induced by the hepatocarcinogen, 2-acetylaminofluoren (2-AAF) (133, 255).

Further study in cultured cells demonstrated that a nuclear factor-kappa B (NF-κB) site (−167 to −158 bp from the transcription start site) on the rat *mdr1b* promoter was required for the induction of *mdr1b* expression by 2-AAF. Overexpression of antisense p65 or IκBβ, an inhibitor of NF-κB, partially abolished the induction. 2-AAF treatment led to the increase of intracellular ROS, which causes activation of IκB kinase (IKK), degradation of IκBβ (but not IκBα), and increase in NF-κB DNA-binding activity. The antioxidant *N*-acetylcysteine (NAC) inhibited the induction of *mdr1b* expression by 2-AAF. Overproduction of GSH by transfection with expressing recombinant encoding its rate-limiting enzyme, γ-GCS large subunit (γ-GCSh), blocked the activation of IKK kinase complex and NF-κB DNA binding. These results strongly suggested that 2-AAF upregulates *mdr1b* through the redox signaling that activates IKK through degradation of IκBβ, and subsequent activation of NF-κB (61) (Fig. 4).

Although the promoter sequences of human *MDR1* and rat *mdr1b* are quite distinct, 2-AAF also induces *MDR1* expression in human hepatica cells through an NF-κB DNA sequence located at −6092 bp. Treating hepatoma cells with 2-AAF activates phosphoinositide 3-kinase (PI3K) and its downstream effectors Rac1 and NADPH oxidase, a cellular source of ROS generator (Fig. 5). Transient transfection assays demonstrated that constitutively activated PI3K and Rac1 enhanced the activation of the MDR1 promoter by 2-AAF. Treatment of hepatoma cells with 2-AAF also activates another PI3K downstream effector protein kinase B (Akt). Transfection of recombinant encoding a dominantly activated Akt also enhanced the activation of *MDR1* promoter activation by 2-AAF. These results demonstrated that 2-AAF upregulated *MDR1* expression is mediated by the multiple effectors of the PI3K/ROS signaling pathway (137).

Hepatocarcinogen DEN, which promotes hepatocarcinogenesis with the accompanying *mdr1a* expression in mice, is also through the NF-κB activating pathway (186). The upstream signaling that activates NF-κB induced by DEN is Toll-like receptor (TLR)-MyD88 in the Kupffer cells. DEN causes 100% HCC in male mice but only 10 to 30% in female mice. The NF-κB signal not only upregulates *mdr1a*, which may contribute to intrinsic drug resistance (see later), but also enhances the production of various cytokines that promote hepatic proliferation. One of these cytokines is interleukin (IL)-6, which is responsible for the gender difference in DEN-associated HCC incidence (186) (Fig. 4).

The results described underscore the importance of the NF-κB transcription factor in the carcinogen-induced *MDR1* gene activation. NF-κB represents a group of structurally related and evolutionarily conserved proteins, with five members in mammals: Rel (c-Rel), RelA (p65), RelB, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) (75). The classic NF-κB activating pathway is activated by a variety of cytotoxic compounds as well as many innate and adaptive immunity mediators such as TNF-α, IL-1β, and Toll-like receptor. The elicited initial signalings all converge in the activation of the IKK complex by phosphorylation of its component IKKβ, which consists of scaffold protein NEMO (also called IKKγ), IKKα, and IKKβ kinases. Once activated, the IKK complex in turn phosphorylates inhibitory protein kappa B (IκBα on Ser32 and Ser36, which is then ubiquitinated and degraded via the proteasome pathway. This releases NF-κB p65/p50, which translocates into the nucleus and activates its target genes. The nonconventional pathway is INK/IKKα dependent and IKKβ independent but involves the phosphorylation and processing of p100, generating p52:RelB heterodimers, which then translocate into the nucleus and activate transcription (Fig. 4). Both path-

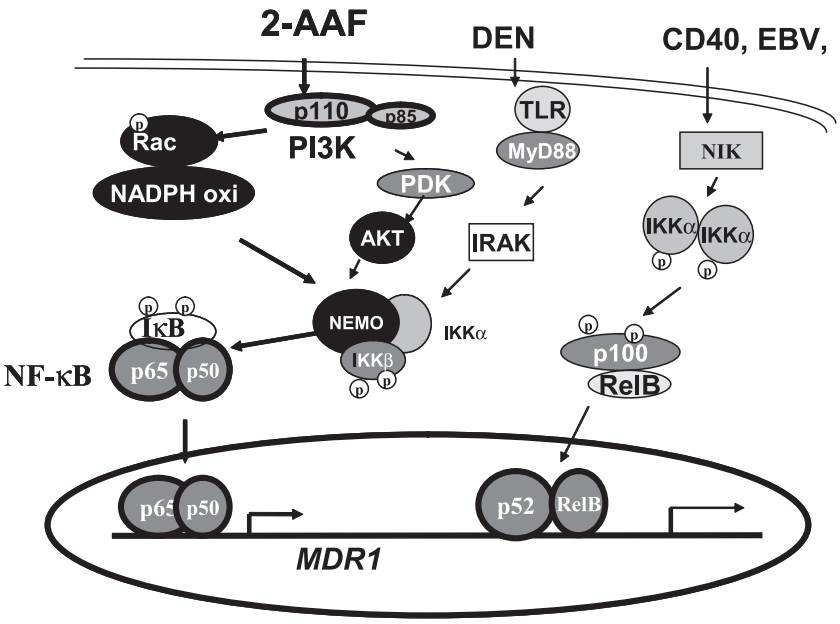

FIG. 4. NF-κB signaling and *MDR* gene expression. Activation of *mdr1b* expression by the carcinogens 2–AAF and DEN is shown in two different pathways but converges into NF-κB signaling. Induction by 2–AAF is through the activation of PI3K signaling, whereas that by DEN occurs through the Toll-like receptor (TLR) signaling pathway. Membrane TLRs recognize external signals. When activated, the majority of TLRs induce activation of NF-κB and cytokine production in a MyD88–mediated manner. Also shown is the nonconventional transduction pathway induced by CD40 and EBV.

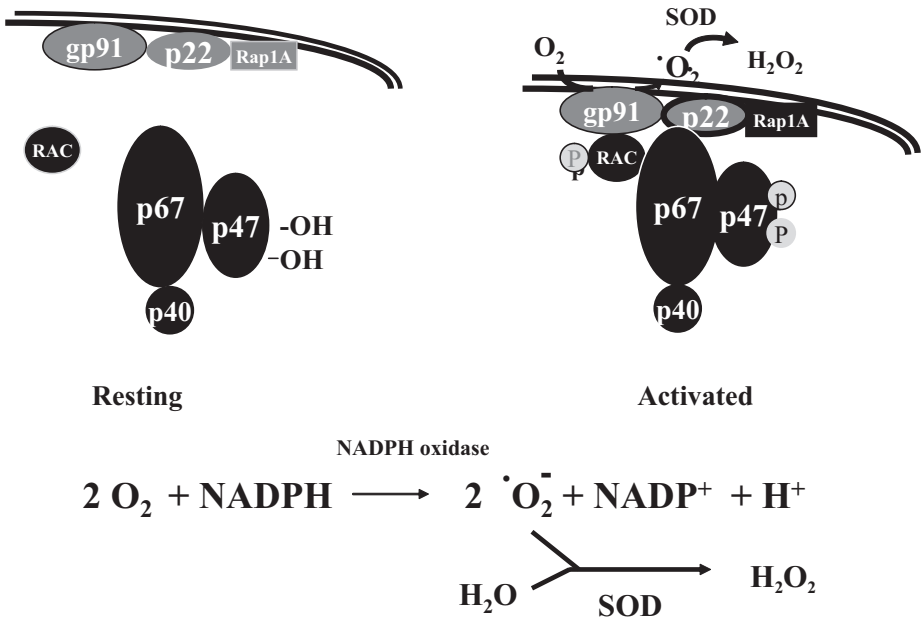

FIG. 5. Activation of NADPH oxidase and the generation of ROS. NADPH oxidase consists of six subunits. Under noninduced conditions, two of the subunits (gp91 and p22) are membrane bound, and the other four (p67, p47, p40, and Rac) are in cytoplasm. Activation is initiated by Rac1 and p47 phosphorylations and brought all the subunits to the membrane, where it generates superoxide, which is metabolized by superoxide dismutase (SOD) into water.

ways ultimately lead to the transcription of distinct sets of target genes, mediating different biologic functions. Apparently, activation of MDR1 expression by 2-AAF and DEN treatments is mediated by the classic IKKβ-dependent pathway.

The Epstein–Barr virus (EBV) is a ubiquitous human herpesvirus and frequently is associated with the development of different epithelial malignancies. Breast cancer cells infected with EBV conferred resistance to paclitaxel with overexpression of *MDR1* (7). Although the mechanism of MDR1 upregulation by EBV infection has not been investigated, it has been demonstrated that EVB can elicit activation of NF-κB signaling through the nonconventional transduction pathway (165) (Fig. 4). It is likely that EBV-induced MDR1 expression in breast cancer cells is involved with the nonconventional NF-κB signaling, but this remains to be investigated.

Antitumor agents: Induction of MDR1 expression by antitumor agents is likely to be an important contributor to the acquired multidrug resistance in cancer chemotherapy. Many studies have shown that *MDR1* gene expression can be transiently induced by cancer chemotherapeutics, and in some studies, transcriptional regulation is involved (see review in 186 and references therein). Of particular importance is that such transient upregulation of MDR1 expression was also seen in chemotherapy. In one acute myeloid leukemia (AML) patient, the Pgp1 level was upregulated *in vivo* at 4 and 16 h after the standard treatment with daunomycin/Arac. Acute induction of *MDR1* gene expression in four of five patients with unresectable sarcoma pulmonary metastases who underwent isolated single-lung perfusion with doxorubicin was seen 50 min after the administration of doxorubicin (2). The mechanisms of upregulation in these patients were not studied.

The expression of *MDR1* in tumors has been evaluated in many clinical investigations in leukemia and in solid tumors. Elevated expression of *MDR1* after chemotherapy has been observed in AML, breast cancer, ovarian cancer, and cervical cancer, suggesting the induction of *MDR1* expression by chemotherapeutic treatments. The mechanism by which *MDR1* expression levels were elevated in the patients was not determined. Other studies, however, showed no changes in *MDR1* expression levels after chemotherapy [for review, see ref. (249)].

Hypoxia: Advanced local tumors often contain areas where oxygen availability is reduced, because of the poor supply of functional blood vessels. The expression of hundreds of genes in cancer cells is altered under hypoxic conditions (234). *MDR1* expression has been shown to be induced by hypoxia. A conserved hypoxic response is mediated by the transcription factor complex known as hypoxia-inducible factor (HIF), which consists of one constitutively expressed HIF-1β (also known as ARNT) and one of the three HIF-1α forms (HIF-1α, HIF-2 α, and HIF-3α). HIF-1α is very unstable under normoxic conditions, whereas it is stabilized under hypoxic conditions. Oxygen regulation of HIF-1α levels lies in the two proline residues of the HIF-1α oxygen-dependent domain, which are hydroxylated by prolyl hydroxylase-domain protein (PHD, also called EglN). Hydroxylated HIF-α is recognized by the von Hippel-Lindau (pVHL) and is targeted for ubiquitination and subsequent proteasomal degradation (99).

Recent results indicate that the stability and expression levels of HIF transcription factors can be regulated by ROS under nonhypoxic and hypoxic conditions [for reviews, see ref. (43, 201, 252)]. Under normoxia, HIF-1α is stabilized by oxidative stress induced by H_2O_2, and antioxidants markedly attenuate HIF-1α protein accumulation, perhaps by the inactivation of the PHD (201). However, the regulation mechanisms of HIF-1α by ROS under hypoxic conditions are complex. Several groups reported that ROS levels are elevated in hypoxia, whereas others reported that ROS levels are decreased in hypoxia [see ref. (252) and references therein]. This discrepancy may lie in basal cell metabolism and the degree and duration of hypoxia experienced. Regardless, several recent studies demonstrated that mito-

chondria function as oxygen sensors and signal hypoxia by releasing ROS. Dysfunction of the mitochondrial electron-transport chain results in impaired hypoxia-induced ROS that is required for hypoxia-induced HIF-α stability (38, 85, 116, 169), through inactivation of PHD by the bound iron (Fig. 6). In addition to VHL-dependent regulation, the PI3K/Akt or protein kinase B (PKB) signaling-transduction cascade has been implicated in the regulation of HIF-1α (21, 79, 261). It is important to note that PI3K is also involved in the regulation of *MDR1* expression (137).

Induction of *MDR1* by hypoxia is mediated by the binding of HIF to the hypoxia-response element located at the promoter of *MDR1* gene (55, 157). In addition, c-Jun NH_2-terminal kinase (JNK) is also known to affect hypoxia-induced *MDR1* expression (55, 157). Further investigation revealed that the JNK pathway functions as a negative regulator of MDR1 expression under normoxia, whereas JNK-induced *MDR1* activation under hypoxic conditions is dependent on increased HIF1 binding to the hypoxia-response element where transcription co-activator p300/CBP also participates (157).

2. Posttranscriptional regulation. Translational control of *MDR1* gene expression has recently been described in K562 myelogenous leukemic cells treated with several different antitumor agents. The half-life of *MDR1* mRNA from naãve K562 cells is ~1 h, whereas that from doxorubicin- and colchicine-resistant cells is 12–16 h (279), suggesting that induction of the steady-state *MDR1* mRNA levels was due to enhanced mRNA stability. Interestingly, this stabilized mRNA was not associated with translating polyribosomes and did not direct Pgp synthesis. However, selection for drug resistance by continuing exposure to the drug led to release of the translational block, MDR1 mRNA translation, and Pgp expression (279). Translational blockage was apparently due to the presence of a double-stranded configuration with loops located at the 5′-UTR of *MDR1* mRNA. When this structure was inserted into a luciferase reporter construct, on transfection of the reporter construct into cultured cells, this structure downregulated the translation efficiency of the reporter under the treatment with a cytotoxic drug. This double-stranded structure affects translation efficiency initiated by eIF4E. This translation repression can be relieved by overexpression of eIF4E (204). These observations demonstrate the translational control of MDR1 protein expression by cytotoxic assaults.

3. Epigenetic regulation. Two types of epigenetic regulation have been discovered in *MDR1* gene regulation. The first one is DNA methylation. The promoter region of *MDR1* is highly GC rich and contains many CpG islands that are targets of DNA methylation-related transcriptional silencing. Several studies have demonstrated that the methylation status in the *MDR1* promoter is correlated with the transcriptional activity of *MDR1* gene (12, 13). The methylation reaction is catalyzed by DNA methyltransferases (DNMTs) using *S*-adenosylmethionine (SAM) as methyl donor to the 5 position of the pyrimidine ring of cytosine within CpG dinucleotides in genomic DNA, resulting in the production of 5-methyl-cytosine and S-adenosylhomocysteine (Fig. 7). Two classes of DNMTs are present in mammalian cells. DNMT1 belongs to the first class and is associated with a replication fork and methylates CpG in the newly synthesized DNA strand. DNMT3 belongs to the second class required for *de novo* methylation during embryonic development. DNMT1 is essential for the maintenance of DNA-methylation patterns in proliferating cells. SAM is the first metabolite of the methionine cycle catalyzed by S-adenosylmethionine synthetase, also known as methionine adenosyltransferase (MAT). The methionine cycle is the major source for cysteine, a precursor of GSH in the transsulfuration pathway. However, intracellular GSH level plays an important role in the maintenance of methylated DNA. Depleting GSH by using the hepatotoxin bromobenzene resulted in reduced intracellular methionine pools and genome-wide DNA hypomethylation (150). Another target in the methionine cycle that is subject to redox regulation is MAT, which exists in three forms in mammalian cells, MAT1, MATII, and MATIII.

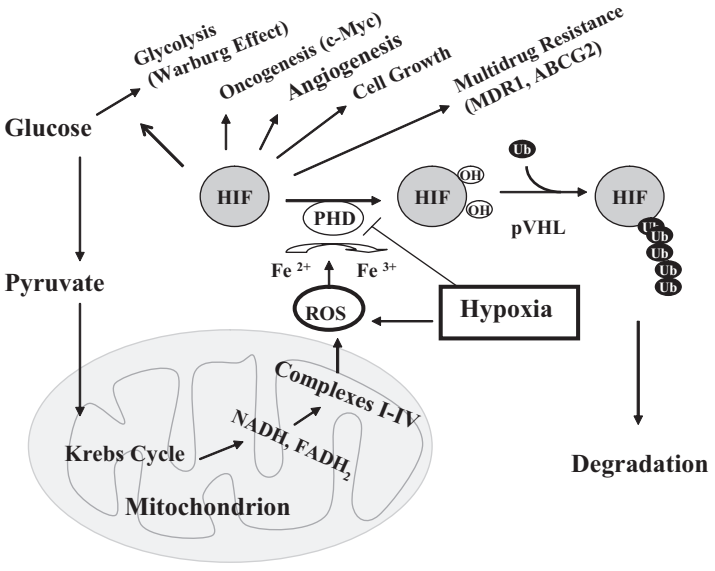

FIG. 6. Redox-regulated HIF-mediated signaling pathways in multidrug-resistance gene expression and other physiologic processes. In the presence of oxygen, the α subunit of the heterodimeric transcription factor HIF is hydroxylated on prolyresidues by PHD. Hydroxylated HIF is subjected to polyubiquitination by pVHL for degradation. PHD requires oxygen and Fe^{2+}. Hypoxia inhibits proly hydroxylation, resulting in stabilization of HIF, and through increased ROS, which oxidize PHD-bound iron. Mitochondrial complexes I to IV are required for hypoxia-mediated ROS. Stabilized HIF elicits multiple physiologic processes, including enhanced glycolysis (the Warburg effect), oncogenesis, angiogenesis, cell growth, and regulation of multidrug-resistance gene expression.

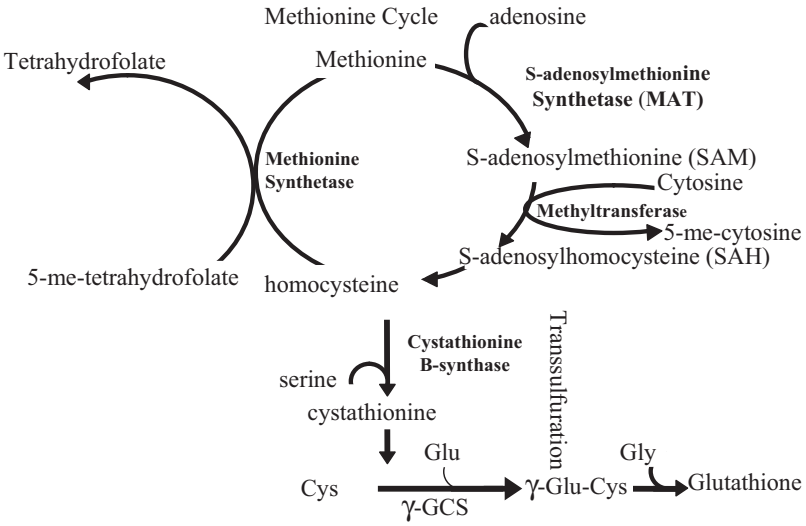

FIG. 7. Schematic diagram showing the effects of redox conditions on the metabolism of the methionine cycle and DNA methylation. A transsulfuration pathway is involved the production of cysteine, which is the substrate for the production of glutathione. SAM, *S*-adenosylmethionine; SAH, S-adenosylhomocysteine; MAT, S-adenosylmethionine synthetase; γ-GCS, γ-glutamylcysteine synthetase; gly, glycine; glu, glutamine; cys, cysteine.

MATI functions as a homotetramer. MATII is the predominant form of SAM synthetase and exists in a multimeric complex. MATIII is a homodimer (86). The activities of these MATs are profoundly influenced by redox conditions, through the maintenance of homotetrameric conformation.

The second type of epigenetic regulation is through histone modification at the *MDR1* promoter chromatin. The gene-silencing effects of methylated DNA are contributed mainly by the binding of the repressor protein, methyl-CpG-binding protein-2 (MeCp2), which has been demonstrated to localize at hypermethylated *MDR1* chromatin. Demethylation of the *MDR1* promoter by 5-azacytidine treatment was associated with release of MeCp2 from *MDR1* promoter and its transcriptional activation. MeCp2 silences transcription by recruiting the interacting co-repressor proteins, SWI independent protein 3 (mSin3), histone deacetylase 1 (HDAC1), and HDAC2. Oxidative damage to CpG islands inhibits binding of the MeCp2 to the lesions (264). Moreover, treatment of cells with the HDAC inhibitors trichostatin A or sodium butyrate leads to the activation of hyperacetylation of MDR1 proximal-promoter histone and activation of MDR1 transcription (114). Increased expression of MDR1 and Pgp was observed in the peripheral blood mononuclear cells (PBMCs) of patients enrolled in a phase I or II trial with depsipeptide, a histone deacetylase inhibitor. Increased levels of histone H3 acetylation were found in PBMCs and circulating tumor cells obtained from patients receiving depsipeptide. Because depsiptide is a substrate of Pgp, these results suggest that upregulation of *MDR1* induces its mechanism of resistance (216). Taken together, these observations underscore the importance of redox in epigenetic regulation of *MDR1* expression in the clinical setting.

B. γ-Glutamylcysteine synthetase (γ-GCS)

γ-GCS is not a multidrug-resistance gene, but its expression is closely associated with that of *MRP1*. As alluded to earlier, the transport activities of several members of the MRP family are regulated by GSH availability, and γ-GCS is the rate-limiting enzyme for the *de novo* biosynthesis of GSH. Co-regulation of γ-GCS and MRP1 would facilitate the efflux activity (Fig. 8). Indeed, as is discussed later, we found that a number of agents that induce the expression of γ-GCSh

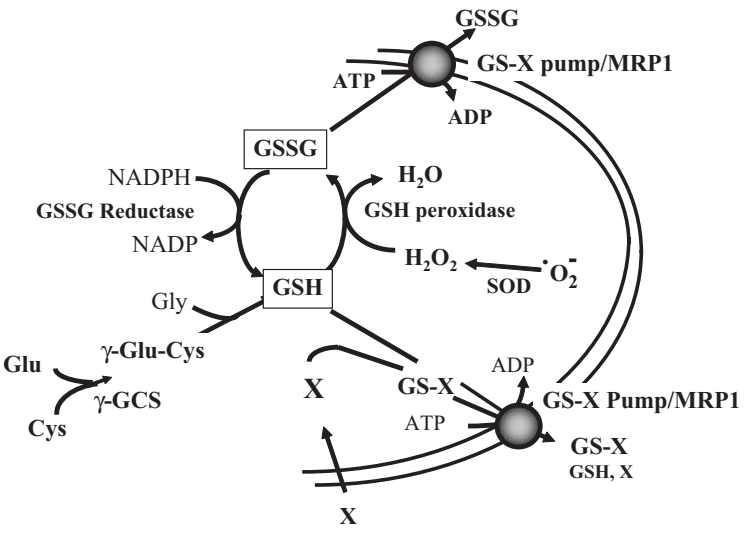

FIG. 8. Roles of GSH in MRP-mediated drug transport. *De novo* biosynthesis of GSH is carried out by γ-GCS, which conjugates glutamine (Glu) and cysteine (Cys) followed by GSH synthetase, by using glycine (Gly) as a substrate. GSH can be oxidized into GSSG by GSH peroxidase. GSSG is a substrate of the MRP/GS-X pump. GSH either forms a conjugate or functions as a cofactor for MRP1/GS-X pump-mediated transport. Thus, GSH by itself can be transported by the MRP1/GS-X efflux pump, albeit at a reduced rate, as compared with that of GSSG. The importance of GSH in MRP1/GS-X–mediated transport is reflected by the frequent co-upregulation of γ-GCS and MRP1 by extracellular influences.

can also induce the expression of *MRP1*. The mammalian γ-GCS is a heterodimer consisting of one 73-kDa heavy or catalytic subunit (γ-GCSh) and one 28-kDa light (regulatory) subunit (γ-GCSl). Hereditary γ-GCSh deficiency is associated with anemia, jaundice, and neurologic abnormalities (87, 213), whereas hepatic ablation of γ-GCSh results in steatosis with mitochondrial injury and hepatic failure, demonstration of the essential roles of GSH in the liver physiology (48). Here I present the mechanisms underlying redox regulation of γ-GCSh, but similar mechanisms may be applicable to the regulation of γ-GCSl.

1. Transcriptional regulation. Our laboratory previously demonstrated that expression of γ-GCSh can be induced by a number of cytotoxic challenges, including antitumor agents (77, 109), heavy metals (109), carcinogens (282), and prooxidants (106, 282). All these inducers, at the concentrations used, exert various degrees of oxidative stresses. Furthermore, enhanced expression of γ-GCSh mRNA was found in colorectal cancers, which are associated with inflammation-associated oxidative stress (136, 251). These observations strongly suggested that the GSH/γ-GCS system is a molecular sensor of oxidative stress conditions. Whereas elevated expression of γ-GCSh catalyzes the enhanced expression of GSH, we observed increased GSH levels feed back and downregulate the steady-state γ-GCSh mRNA level (282). This feedback mechanism underscores the importance of γ-GCSh as a major redox regulator.

Transcriptional regulation of γ-GCSh expression is mediated by an antioxidant response element (ARE) that contains a consensus sequence 5′-TGAGTCA, which interacts with NF-E2–related factor (Nrf2). In *Nrf2*(−/−) animals, expression levels of γ-GCSh mRNA and γ-GCSl mRNA are only 58% and 65%, respectively, of the level in wild-type controls (42), demonstrating the role of Nrf2 in the regulation of steady-state levels of γ-GCSh and γ-GCSl.

The transcription factor Nrf2 is a member of the "cap 'n' collar" family of basic leucine zipper transcription factors. Mechanisms by which Nrf2 mediates upregulation of antioxidant enzyme genes have been the subjects of intensive study in recent years (56, 290). Nrf2 binds to ARE and regulates ARE-mediated antioxidant enzyme genes both at the basal levels and in conditions in which upregulation is stimulated by a variety of antioxidants. Nrf2 is an unstable protein, the stability of which can be enhanced by the treatment with proteasome inhibitor lactacystin. This treatment resulted in increased steady-state levels of γ-GCSh mRNA levels (231). Under nonstress conditions, Nrf2 is bound to Kelch-like ECH-associated protein (keap1), which is a cytosolic actin-associated protein (112). Keap1 functions as a substrate adaptor for a Cullin-dependent E3 ubiquitin ligase complex and targets Nrf2 for ubiquitination after proteasomal degradation (Fig. 9). Human Keap1 contains 27 cysteines of a total of 624 amino acids. Cys273 and Cys288 are essential for the repressive activity of Keap1 under nonstress conditions (127). Under oxidative stress conditions, Keap1 undergoes a Cys151-mediated posttranslational modification, resulting in a conformational change of the Keap-Cullin3 (Cul3)-Rbx1 E3 ubiquitin ligase. This conformational change suppresses the ubiquitin ligase activity, thereby inhibiting Nrf2 degradation (238) and allowing Nrf2 to translocate into the nucleus and heterodimerize with the small Maf proteins, MafK or MafG

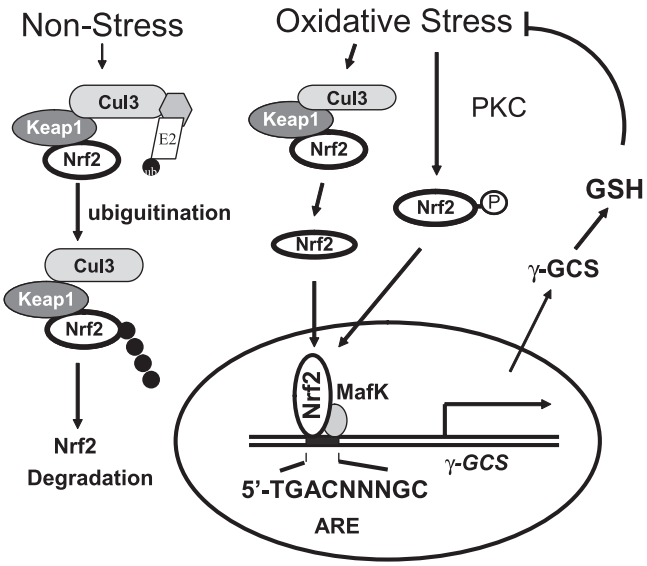

FIG. 9. Nrf2 and transcriptional regulation of γ-GCSh expression. Under nonstress conditions, Nrf2 is complexed with Keap1 and targeted to Cul3–mediated ubiquitination and protein degradation. Under oxidative-stress conditions, such a degradation pathway is inhibited, and Nrf2 accumulates. Nrf2 then translocates into the nucleus, where it complexes with MafK, which transactivates target genes. Oxidative stress also induces Nrf2 phosphorylation. *γ-GCSh* is a target gene of Nrf2. Overexpression of *γ-GCSh* leads to elevated biosynthesis of GSH, which in turn suppresses oxidative stress.

co-activator, the basic leucine zipper transcription factor of the Maf (proto)-oncogene family (62). Together they bind to ARE and transactivate γ-GCSh expression. Upregulation of γ-GCSh expression results in feedback to suppress the Nrf2-Keap1 signaling (Fig. 9). Similar mechanisms are involved in the regulation of the expression of many so-called phase II detoxifying enzymes [*e.g.*, quinine oxidoreductase 1, glutathione S-transferase (GSTA1), and heme oxygenase 1 (188)], which covalently modify xenobiotics for elimination by transport.

Additional pathways have been described to account for induced Nrf2-mediated transcriptional activation.

1. Oxidative stress activates several protein kinases including PKC (28, 101), p38 mitogen-activated protein kinase (287, 294) and PI-3 kinase (144), which phosphorylate Nrf2 and enhance its transactivating signaling of ARE containing genes.
2. Oxidative stress disrupts keap1-Nrf2 interactions by modifying the two critical cysteine residues of keap1, resulting in the release of Nrf2, which subsequently translocates into the nucleus (112) (Fig. 9).
3. An additional mode of regulation of Nrf2 is the subcellular localization of Keap1. Although the primary distribution of Keap1 is in the cytoplasm, it has been observed to accumulate in the nucleus in the presence of leptomycin B, an inhibitor of nuclear export signal. Keap1 does not contain a nuclear-localization signal amino acid sequence, but Nrf2 does. It is suggested that Keap1 may ride on Nrf2

and shuttle in and out of the nucleus. It has been suggested that under normal physiologic conditions, Nrf2 is the target for Keap1-induced proteasome-mediated degradation. Oxidative stress prevents Keap1 from targeting Nrf2 to proteasome degradation inside the nucleus, resulting in further nuclear accumulation of Nrf2 and transactivates ARE-mediated gene expression (187).

2. Posttranscriptional regulation. In addition to the transcriptional regulation of γ-GCSh expression mediated by the transcription factor Nrf2, oxidative stress can regulate gene expression posttranscriptionally. Posttranscriptional regulation of γ-GCSh expression was previously suggested by nuclear run-on assay (77). Further investigation demonstrated that oxidative stress–inducing agents (*e.g.*, sulindac, pyrrolidinedithiocarbamate, and *tert*-butylhydroquinone), induced γ-GCSh mRNA stabilization through the p38 MAP kinase pathways. P38 MAP kinase activates MAPKAPK2, which promotes the cytoplasmic translocation of mRNA-stabilizing factor HuR, a member of ELAV family of AU-rich binding RNA-binding protein, from the nucleus to cytoplasm and interacts with the AU-rich sequence located at the 3′ untranslated region of γ-GCSh mRNA. The accumulated γ-GCSh mRNA produces elevated levels of GSH, which suppress oxidative stress and also feeds back to suppress γ-GCSh mRNA stabilization (242) (Fig. 10).

The posttranscriptional regulation mechanism of γ-GCSh mRNA levels may have clinical implications. It has long been thought that increased oxidative stress is associated with carcinogenesis, particularly in colon cancer (41). It was previously observed that γ-GCSh is frequently overexpressed in colon cancer (136, 251). Moreover, the development of colon cancer in experimental animals correlates with HuR content. Colon cancer cells overexpressing HuR produced significantly larger tumors than those arising from control cells, whereas those with reduced HuR levels through small interference RNA or antisense HuR-based approaches developed significantly more slowly (163). Last, constitutively elevated p38 MAPK expression is frequently associated with cancer progression (63, 171, 173).

Activation of p38 MAP kinase signaling by oxidative stress can be traced to the upstream regulator, MAP kinase kinase kinase (MKKK). At least one mechanism can account for the activation of MKKK by oxidative stress. It has been demonstrated that under normal physiologic conditions, the reduced form of thioredoxin is complexed with apoptosis signal-regulating kinase 1 (ASK1) (also known as MKKK 5) and inhibits ASK1 activity (105). With oxidative stress, thioredoxin is oxidized and dissociated from ASK1, which subsequently activates downstream signaling, by sequential phosphorylation of MKK3/6 and p38 MAPK (80) (Fig. 10). In addition, many antioxidative enzymes and molecular chaperons, including HPs72, glutaredoxin, and glutathione *S*-transferase, also inhibit the activation of ASK1 (170).

Another mechanism by which ROS activate MAPK pathways is by inactivating phosphatases, such as protein tyrosine phosphatase 1B and protein phosphatase C2 and C5 (123). Overexpression of GSTm1 has been reported to inhibit the oxidative stress–induced activation of p38 MAPK by binding to and inhibiting ASK1 (51). Dolado *et al*. (64) reported that p38α-deficient cells derived from *p38α−/−*) mouse, when transformed by the activated HRasV12 oncogene, caused increased proliferation, increased number of foci in soft agar, ability to form colonies in soft agar, and decreased apoptosis (64). These effects could be rescued by ectopic expression of p38 MAPK. This observation suggests that p38 MAPK functions as a suppressor of tumor formation by HRas, which is known to cause increased ROS production. Dolado *et al*. (64) showed that the accumulated ROS in the HRas-transformed fibroblasts induce apoptosis, whereas the p38α-deficient cells are resistant to the ROS-induced apoptosis. These observations suggest that the ability

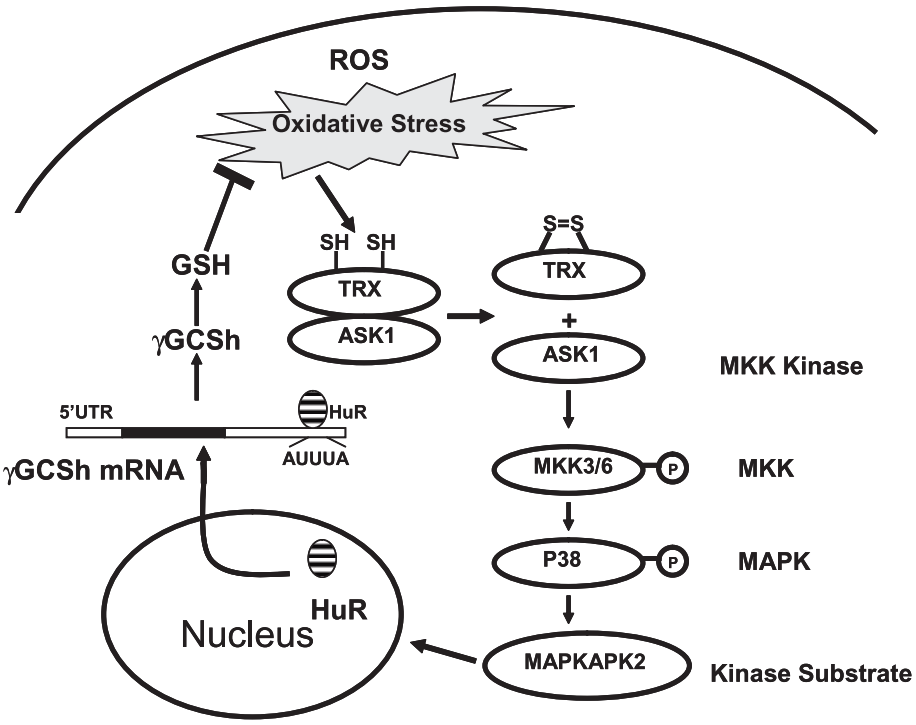

FIG. 10. Posttranscriptional regulation of γ-GCSh mRNA stability by the MAP kinase pathway under oxidative-stress conditions. Oxidative stress activates MKK kinase, which phosphorylates its downstream mediators, MKK, MAPK, and MAPKAPK2, resulting in the translocation of mRNA stabilizing factor, HuR, from the nucleus into the cytoplasm. HuR binds to the 3′-UTR of γ-*GCSh* mRNA and stabilizes it from degradation. Increased γ-*GCSh* leads to elevation of an amount of GSH, which in turn suppresses oxidative stress.

of p38 MAPK to activate apoptosis in response to oncogene-induced ROS accumulation is important for the regulation of malignant transformation.

C. MRP family

As with γ-GCSh, our laboratory has demonstrated that expression of *MRP1* in cultured cells can be induced by a variety of cytotoxic agents, including prooxidants (155), heavy metals (109), carcinogens (282), antitumor agents, and nitric oxides (106). These observations raise an interesting scenario that mechanisms underlying induced expression of MRP1 and γ-GCSh may be co-regulated. It should be pointed out that, although frequent simultaneous upregulation of *MRP1* and γ-GCSh has been observed under oxidative-stress conditions, the magnitude and time course of upregulation were not coordinated, depending on the cell lines used (p53 status) and types of inducers (155). This is also true for the induction of other members of MRP gene family. Some members (*MRP3* for example) are more sensitive to induction of certain peroxidants than are others (*MRP1*) (155).

As mentioned earlier, regulation of γ-GCSh gene expression by cytotoxic assaults is mediated at multiple levels, including transcriptional and posttranscriptional regulations. Whereas transcriptional regulation of γ-GCSh is mediated by Nrf2, which recognizes ARE in the promoter of γ-GCSh, no such element has yet been identified in the promoters of human *MRP1* and *MRP2* (122, 250). However, when Nrf2 was knocked down by siRNA, induction of *MRP2* and *BCRP*, but not *MRP1*, by *tert*-butylhydroquinone, in human hepatoma HepG2 cells was significantly suppressed (3). These results demonstrate that although expression of both *MRP1* and *MRP2* can be induced by oxidative stress, only *MRP2* expression is Nrf2 dependent.

The potential involvement of Nrf2 in the regulation of murine *mrp1*, *mrp2*, *mrp3*, and *mrp4* has been suggested, but the results are not consistent from different reports. Hayashi *et al*. (91) reported that the constitutive expression levels of murine *mrp1* mRNA were significantly lower in *Nrf2*(−/−) cells than in the wild-type cells. Induction levels of *mrp1* expression by diethyl maleate in *Nrf2*(−/−) cells were significantly reduced, suggesting a role of *Nrf2* in the regulation of murine *mrp1* by oxidative stress. An ARE-like sequence that responds to chemical-induced oxidative stress located at the mouse *mrp2* promoter has been identified. This ARE contains an Nrf2 binding site, and expression of Nrf2 upregulates *mrp2* expression (269). *Nrf2*-null mice are very sensitive to the acetaminophen (APAP)-induced hepatotoxicity, resulting in elevated expression of NADPH quinine oxidoreductase (Nqo1) and γ-GCSh. Treatment of *Nrf2*-null animals with APAP showed only moderate reduction of mrp1 and mrp2 proteins. In contrast, significant induction of *mrp3* and *mrp4* mRNA levels by APAP was seen in the wild-type animals but not in the *Nrf2*(−/−) mice, suggesting that Nrf2 is a more potent regulator for the upregulation of *mrp3* and *mrp4* by APAP than for the *mrp1* and *mrp2* in animal studies (4).

In addition to Nrf2, recent results suggested that *MRP1* and *γ-GCSh* are regulated by N-*myc*. The promoters of human *MRP1* and *γ-GCSh* contain multiple E-box sequences that are recognized by oncoprotein transcription factors N-*myc* and C-*myc*. In childhood neuroblastoma, high levels of MRP1 expression correlate with overexpression of N-*myc*. Enforced expression of N-*myc* by using a tet-inducible expression system increased *MRP1* expression, resulting in increased drug resistance and enhanced *MRP1*-mediated drug efflux (168). Likewise, it has been demonstrated that *γ-GCSh* is one of the target genes of c-*Myc* (22). Oxidative stress induced by H_2O_2 treatment recruits c-*Myc* to the *γ-GCSh* promoter through ERK-dependent phosphorylation at Ser62 (22, 23). These studies may also explain the coordinated expression mechanism of *γ-GCSh* and *MRP1* by oxidative stress and also provide a clue as to how expression of C-*myc* may regulate intracellular ROS levels that contribute to genomic instability, cell-cycle regulation, and cell proliferation.

D. BCRP/ABCG2

The initial discovery that pheophorbide *a* is an endogenous substrate for *BCRP* revealed the importance of intracellular porphyrin/heme contents regulation by *BCRP* (115). The regulation of porphyrin and heme in the context of redox biochemistry is important for the following reasons: (a) The iron contained in heme serves as a source of electrons during electron transfer in redox biochemistry. Accumulation of heme within the cell ultimately leads to iron overload, resulting in the production of ROS by the Fenton reaction; and (b) Heme/porphyrin accumulation also leads to the collapse of mitochondrial function, the powerhouse for generation of ROS. This is particularly acute when cells are exposed under hypoxic conditions in which oxygen tension is low.

Biosynthesis of porphyrin/heme is initiated inside the mitochondrion with the formation of D-aminolevulinic acid (dALA) from glycine and succinyl-CoA from the citric acid cycle (Fig. 11). Two molecules of dALA then form a porphobilinogen catalyzed by ALA porphobilinogen, which, after a few enzymatic reaction steps, leads to the formation of coproporphyrinogen III, all in the cytoplasmic compartment. Coproporphyrinogen III is transported into mitochondrion by the ABCB6 importer (129). After a few intermediate steps inside the mitochondrion, the main end product is protoporphyrin IX, which is combined with iron to form heme. Cellular levels of porphyrin and heme are regulated by the expression of ABCB6, but the elimination of porphyrins is carried out by BCRP/ABCG2. Degradation of heme uses the NADPH/NADP system and requires molecular O_2 with the formation of end-product bilirubin, which is then glucuronided and eliminated by MRP2 in the livers. Therefore, three ABC transporters are involved in the regulation of heme/porphyrin homeostasis, communicating between mitochondrial and cytoplasmic compartments that are tightly regulated by intracellular redox conditions.

The redox biochemistry contributed by porphyrins is their sensitivity to photodynamic excitation. Photoexcited porphyrins play an important role in the formation of singlet oxygen ($^1O_2$) by transferring energy to molecular oxygen, which at ambient temperature behaves as a triplet and is paramagnetic. $^1O_2$ is a strong oxidant owing to its unpaired electrons with opposite spins, whereas the ground-state triplet oxygen has the same spins. $^1O_2$ is an important source for the formation of ROS. Porphyrins-containing proteins play important roles in various physiologic processes, including oxygen transport and storage (hemoglobin), drug

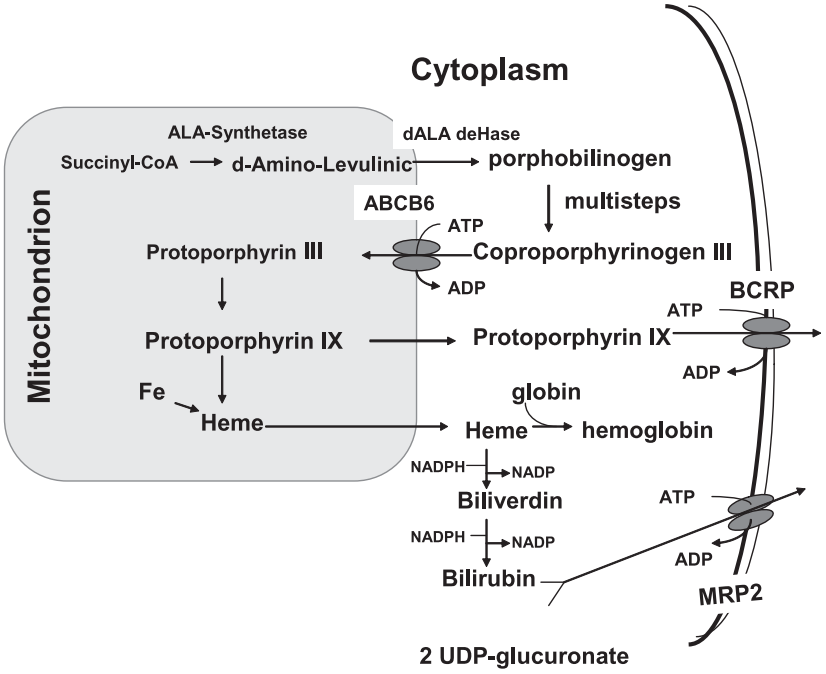

FIG. 11. Metabolic pathways of heme/porphyrin biosynthesis and elimination by ABCG2 transporter. Biosynthesis of protoporphyrin and heme starts in mitochondria. After multiple steps of enzymatic reactions, as indicated. ABCB6 is involved in the import of coproporphyrinogen into the mitochrondrion. Elimination of protoporphyrin IX involves ABCG2. Heme is metabolized to bilirubin, which is eliminated by ABCG2 in the liver. ALA, aminolevulinic acid.

metabolism (CYP450), mitochondrial respiration system (cytochrome *c* oxidase), *etc*. Porphyrias are inherited disorders resulting from the dysfunction of specific enzymes involved in heme biosynthesis (141).

To deal with the build-up of porphyrins, animal cells have developed detoxification mechanisms by increasing the expression of *ABCB6* and *BCRP*. However, mechanisms by which elevated porphyrins enhance the expression of *ABCB6* have not yet been elucidated. Expression of *BCRP* is regulated by hypoxia (107), which is mediated by HIF-1. An HIF-1 interacting *cis*-element is located at the *BCRP* promoter (at −116 to −112 nt) (128). Cells lacking HIF-1 were unable to upregulate *BCRP* expression. Because mitochondrial ROS has been implicated as a sensor for HIF-1 transcription factor, and because mitochondrial ROS also plays an important role in the regulation of porphyrin metabolism, these make mitochondrial physiology an integral partner in the overall regulation of the *BCRP* transporter.

Expression of *BCRP* is also under hormonal regulation. An estrogen receptor–binding element at −190 to −171 nt (66) and a peroxisome proliferator-activated receptor γ (PPARγ)-responsive element (−3946 to −3796) (245) have been located. These *cis*-acting elements are involved in the regulation of *BCRP* by their respectively ligands. Moreover, a recent study demonstrated that transcriptional regulation of *BRCP* expression in the drug-resistant variants is controlled by histone acetylation/deacetylation (258), demonstrating that epigenetic mechanisms also play a role in this regulation.

BCRP is normally localized in the plasmic membrane and intracellular compartment. Recent studies have shown that the PI3K/Akt signaling can affect the distribution of *BCRP* between the apical surface of the membrane and the cytoplasmic compartments. Takada *et al*. (247) demonstrated that an inhibitor to PI3K, worthmannin, causes translocation of *ABCG2* to the intracellular compartment in human LLC-PK1 cells. Redistribution of *ABCG2* was also observed in Akt-dominant negative cells. Similar results were observed in hematopoietic stem cells, where BCRP is enriched in the side population (SP) cells. Bone marrow from Akt-null mice exhibited a reduced SP fraction, and lentivirus-mediated overexpression of Akt1 in bone marrow greatly increased the SP fraction (179). These results demonstrate that the PI3K/Akt signaling pathway modulates intracellular distribution of BCRP; however, the physiologic implication of such redistribution is yet to be defined.

VI. Redox Regulation of Multidrug-Resistance Transporters: Gene Amplification

Amplification of *mdr1* gene copy number was discovered in multidrug-resistant cell lines overexpressing the encoded Pgp1 protein soon after the cloning of the *MDR1* cDNA, and was considered one of the major mechanisms for the regulation of drug resistance (26, 174, 227, 253). Like in many other prior drug-resistant variants, amplified DNA can be either extrachromosomally located, known as double minutes (DM), or chromosomally borne at segments manifested as abnormal banding regions (ABRs). For reasons yet to be determined, amplified *MDR* genes in drug-resistant murine and human cell lines are often manifested as the DM configuration, whereas in Chinese hamster cells, they appear as ABR (138).

In early observations, ABR-containing amplified DNA in hamster drug-resistant variants were often localized distal to the single-copy locus on the same chromosome (138) (Fig. 12B). The underlying mechanism of this cytogenetic manifestation of DNA amplification is consistent with the break-fusion-bridge (B-F-B) mechanism. According to this model (Fig. 12A), an initial isochromatid break distal to the target gene of a specific cytotoxic agent produces uncapped chromosomes. After isochromatid fusion and replication, the resulting dicentric chromosome moves toward two opposite poles during the telophase of the cell cycle. An additional

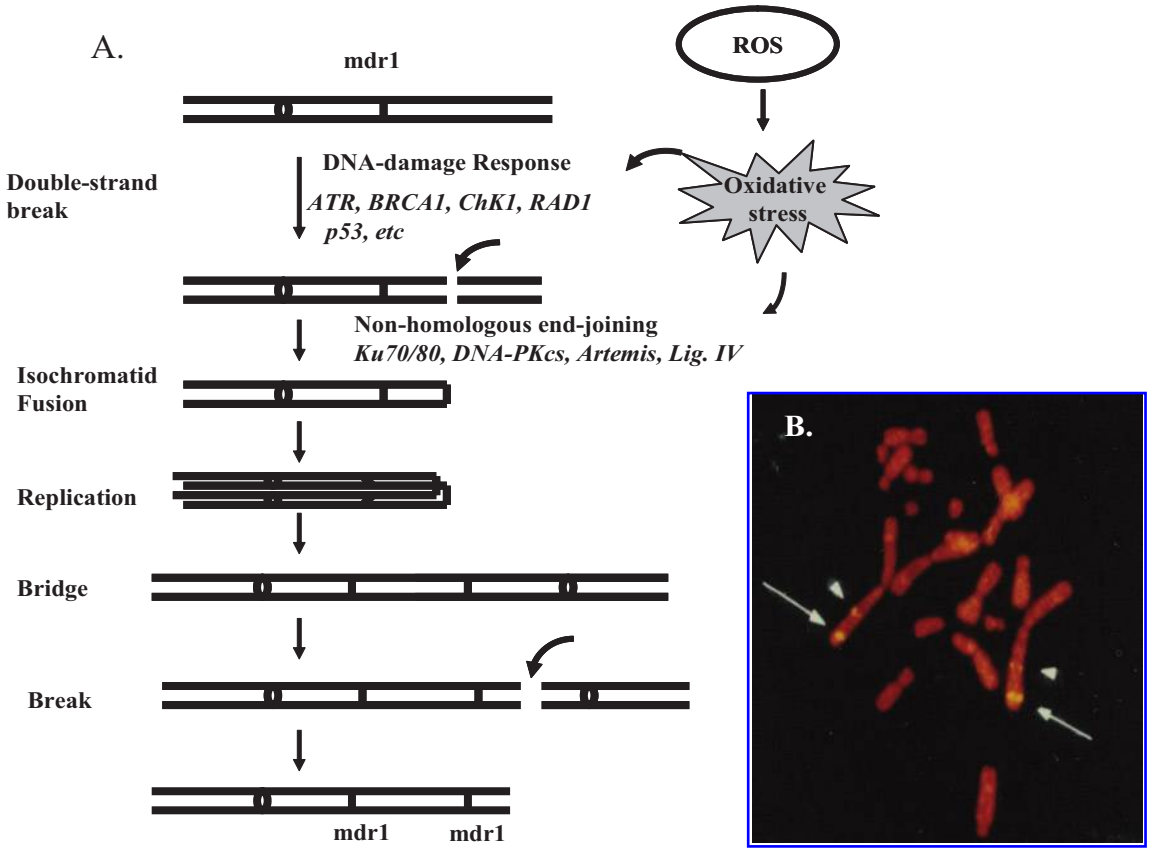

FIG. 12. Effects of ROS on amplification of the *mdr1* gene. (**A**) Schematic diagram showing the break-fusion-bridge (B-F-B) mechanism of amplification of the *mdr1* gene in MDR CHO cells. The effects of ROS on factors that regulate chromosomal breakage and isochromatid fusion are indicated. (**B**) A photograph of fluorescence *in situ* hybridization showing the locations of single-copy *mdr1* (*arrowheads*) and amplified *mdr1* (*arrows*) on the Chinese hamster chromosomes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this artilce at www.liebert-online.com/ars).

break between the centromere and the target gene of the dicentric chromosome occurs. The resultant chromosome contains a duplicated chromosomal segment with inverted polarities that are bordered by the target (single-copy) gene and the distally located amplified gene (Fig. 12A). This rather consistent chromosomal abnormality is found in many Chinese hamster drug-resistant cell lines (138). Chinese hamster *mdr1a* is located at 1q26 and the amplified *mdr1a* clustered at 1q32, distal to the single copy locus. Similar results also were observed in *MDR* cell lines. *BCRP* is located at chromosome 4q21-4q22, and amplified *ABCG2* sequence peaks at 4q21-4q22 in multidrug-resistant variants with acquired resistance to *ABCG2* drug substrates (65).

This B-F-B model of DNA amplification underscores the importance of the initial chromosomal break that triggers the B-F-B cycle. Kuo *et al*. (139) observed that in three independently established MDR-CHO cell lines selected with vincristine, vinblastine, and Adriamycin, the break sites are all located on chromosomal 1q31. Because the cytotoxic agents used in the selection of *MDR* cells exhibit different modes of cytotoxic mechanism yet gave rise to the same location of chromosomal breakage, it was hypothesized that the breakage site is likely to be a chromosomal fragile site, and the initial event that triggers the B-F-B cycle is a stress-induced breakage at the fragile sites. This hypothesis was indeed demonstrated; CHO 1q31 is a major fragile site and is sensitive to common fragile-site inducers, methotrexate and aphilicolin. The role of major chromosomal fragile sites in initiating DNA amplification was confirmed in many other drug-resistant variants, as well as in human tumor cell lines harboring amplified oncogenes with growth advantages (92, 160, 194, 238, 243). DNA amplification mediated by the B-F-B mechanism underscores two rate-limiting steps: the initial chromosomal breakage at the fragile site and the sister chromatid fusion. Both steps are modulated by redox signaling.

A. Role of redox signaling in chromosomal breakage at fragile sites

Chromosomal fragile sites have been traditionally considered loci that are intrinsically sensitive to cytotoxic assaults (135). Many cytotoxic agents used in *MDR1* DNA-amplification studies such as vincristine, vinblastine, puromycin, and actinomycin D, have no known mechanism of action directly on chromosomal DNA. Therefore, the breakage associated with the DNA amplification initiated at the fragile site must be considered as secondary to the primary targets of these agents, consistent with the hypothesis of stress-induced cellular response (135).

Isochromatid breaks at chromosomal fragile sites produce one broken chromosome without a telomere and one acentric chromosome fragment. The latter will eventually disap-

pear after multiple runs of cell division. Chromosomes without telomeres, if uncapped, are unstable and prone to chromosomal abnormalities, including deletion, rearrangement, translocation, and fusion. Chromosomes that have lost a telomere may eventually be stabilized by *de novo* addition of a new telomere (11). The B-F-B cycle is observed only in cells defective in cell-cycle checkpoints, such as those with a p53-deficient background. This may explain why DNA amplification in drug-resistant tumor cells occurs at a frequency of $\sim 10^{-3}$–10^{-4}, whereas the frequency is much lower (10^{-8}–10^{-9}) in wild-type p53-containing cells (159).

Numerous studies have suggested that oxidative stress that generates free radicals can induce DNA damage and genomic instability. Early studies from Schimke's group demonstrated that frequency of DNA amplification was increased in cells under stress conditions, such as pretreatments with UV (257), hydroxyurea (35), or hypoxia (211). Antioxidants NAC and GSH have been shown to suppress oxidative stress–induced DNA damage (207). Moreover, overexpression of C-*myc* has been tightly associated with the production of ROS, promotion of genomic instability, and tumorigenesis (263). Likewise, overexpressing antioxidant superoxide dismutase (SOD1) in transgenic mice increases chromosome breakage (118). These results provide evidence supporting the role of ROS in chromosomal breakage.

B. Role of redox signal in sister chromatid fusion

Isochromatid fusion is one of many repair mechanisms for DNA double-strand break (DSB). Sister chromatid fusion involved in the B-F-B model predicts the generation of an inverted repeat DNA sequence franking the initial breakpoint. This is indeed the case found in drug-resistant cell lines containing amplified DNA (104, 259). In other studies using MDR cell lines containing highly amplified genes, no regions of extensive homology or inverted repeat sequences were identified at the amplicon junctions (146, 191). The formation of these junctions may be initiated by nonhomologous DNA end-joining (NHEJ) mechanism, or using the microhomology of DNA sequence at the junction (191).

NHEJ is the primary repair mechanism for DSB, which is error prone. In an artificial system where DSBs were generated by site-specific restriction enzyme cleavage (SecI), it was found that the frequency of NHEJ was ~1,000 times higher than that of homologous recombination, which uses a homologous DNA template for accurate repair. NHEJ pathway requires several factors, including a Ku70-86 complex for initial recognition of the DNA break, DNA protein kinase (DNA-PK) for activating repair protein at the breakage site, Artemis for potentiating DNA end-processing enzymes, and XRCC4-Ligase IV complex for religation of the broken DNA ends (40). At least some of the components in the NHEJ pathway are redox sensitive. For example, the function of the Ku complex is functionally regulated by redox mechanisms. Ku86(−/−) animals died during the postnatal weeks, but treating these Ku86(−/−) mice with antioxidant NAC prolonged the survival (208). Likewise, treatment with the antioxidant tempol increases survival of Ku70(−/−) cells in cultures (185). These results demonstrated that a defective Ku70-86 complex enhanced oxidative stress. The activities of the DNA-PK catalytic subunit are also regulated by redox conditions. Oxidative stress induced by the antitumor agent chlorambucil in cultured ovarian cancer cells decreased DNA-PK catalytic subunit activity, whereas pretreating cells with antioxidant NAC increased DNA-PC activity by phosphorylating the threonine 2609 residue (10), demonstrating the reversibility of redox effects on DNA-PK activities. However, the roles of Ku70-86 and DNA-PKsc on sister chromatid fusion require further investigation.

VII. Redox Signaling in the Evolution of Intrinsic Multidrug Resistance in Cancer

A. Evolution of intrinsic multidrug resistance (upfront resistance)

As alluded to earlier, the expression of many multidrug transporters is inducible by oxidative stress. Moreover, many studies have shown the potential relation between the risk of carcinogenesis and ROS (256). A recent study demonstrated that elevated ROS production due to a genetic defect in mitochondrial respiratory complex 1 enhances tumor progress in an animal model (107). Thus, it is not surprising that elevated expression of these drug-resistance genes is found in tumors before chemotherapy. This intrinsic drug resistance has been found in various solid tumors, particularly in liver cancers, where *MDR1* gene upregulation is frequently observed (Fig. 13).

Here, I use *mdr1* gene expression in hepatocarcinogenesis as an example (Fig. 14). The liver is the body's major detoxification reservoir. It has a dual blood-supply (the hepatic artery and portal vein) system, enabling it to receive nutrients and toxic substances from virtually the entire body. The surface of hepatocytes contains abundant receptors, transporters, and carrier proteins that import a wide variety of xenobiotics, including plant alkaloids, antibiotics, and phytotoxins, and industrial toxic byproducts into the hepatocytes intracellularly. It is well known that liver cancer in animals can be induced by a wide variety of signals elicited by carcinogens, alcohol consumption, toxins, metals, and viral infections. Although these various agents have different cellular targets and different modes of cytotoxic effects, their common denominator is the formation of ROS, suggesting that oxidative stress plays an important role in hepatocarcinogenesis. Oxidative stress exerted by cytotoxic agents can also induce antioxidative defense mechanisms, including upregulation of antioxidant enzymes (SOD1, γ-GCSh, GPx, TrxR, catalase, *etc*.) and phase I [cytochrome P450, NAD(P)H quinine oxidoreductase] and phase II (GST, UDP glycosyltransferase) drug-metabolizing enzymes. These antioxidative defense mechanisms feed back to suppress the induced oxidative stress. Prolonged exposure to these cytotoxic agents produces persistent oxidative stress beyond the antioxidative stress that would lead to the development of liver cancers. Concomitantly, elevated expression of the *mdr1* gene is commonly observed in the hepatocellular carcinomas developed in these animals (256) (Fig. 14).

The implication that intrinsic drug resistance is present in human tumors can be found in the recent publication by Bracht *et al*. (33). These investigators showed that in two panels of human tumor cell lines, a 14-cell panel from this group and a 30-cell panel from the NCI, no correlations between the expression of *MRP1* and expression/activity of various antioxidative enzymes such as GPx, TrxR, GR, and GST, as

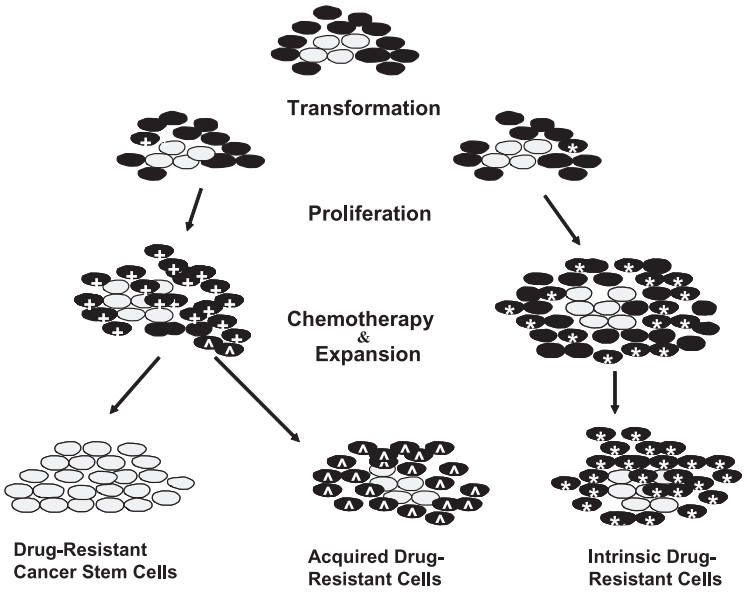

FIG. 13. Hypothetical model depicting the evolution of drug-resistant variants in cancer cells. Drug-resistant populations can be evolved from (a) cancer stem cells, which are intrinsically resistant to chemotherapy (*open circle*), (b) acquired drug-resistant cells (^) after eradicating the drug-sensitive cells (+) by chemotherapy, and (c) from intrinsic drug-resistant cells (*) that involve intrinsic upregulation of drug-resistance genes along with tumor development.

well as GSH levels, was found. Although expression of *Pgp1* and *MRP2* were not detectable, expression of *MRP1* were detectable in 10 of the 14 cell lines. Expression of *MRP1* in these cell lines correlates positively with resistance to vinblastine and etoposide, the substrates of the *MRP1* efflux pump. Many of these *MRP1*-expressing cell lines were from patients who had not been treated with chemotherapy, suggesting a role of *MRP1* in the intrinsic drug resistance.

B. Signal-transduction pathways in the intrinsic multidrug resistance

The signal-transduction pathways that regulate multidrug-resistance gene expression were discussed earlier. What follows is how these pathways interface between multidrug-resistance gene expression and carcinogenesis. Examples of these pathways are discussed.

1. The NF-κB pathway. An important transcriptional activation pathway involved in the upregulation of multidrug resistance is the NF-κB pathway. Activation of NF-κB blocks programmed cell death induced by numerous cytotoxic agents, including chemotherapeutic agents, ionizing radiation, and cytokines. These cytotoxic agents induce ROS. Several studies indicate that NF-κB induces a spectrum of gene expression to counter the accumulation of ROS (195). The cytoprotective measure of NF-κB induced by cytotoxic agents such as doxorubicin is mediated by its downstream transcription mediator, Twist (196).

Activation of NF-κB, as measured by the DNA-binding activity of transcription has been seen in many types of cancers. Several hundred NF-κB target genes are present in the human genome, and many of them are involved in the immune responses, inflammation, apoptosis, cell proliferation, and angiogenesis. Persistent activation of NF-κB has been

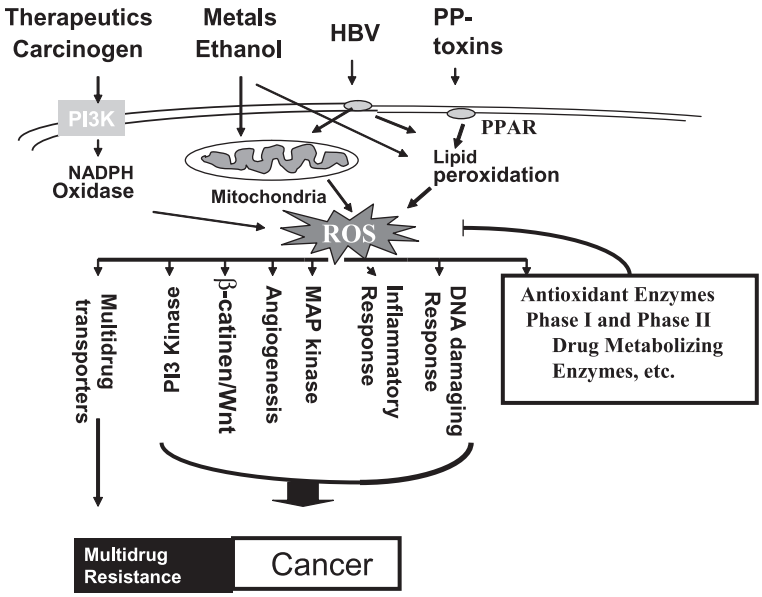

FIG. 14. Schematic diagram showing redox-regulated signal-transduction pathways that are important for tumorigenesis and for the evolution of multidrug resistance in tumors. Cytotoxic insults by various agents produce ROS, which are important regulators for various signal-transduction pathways that are implicated in tumorigenesis. ROS also are important in the regulation of multidrug-resistance gene expression, contributing to the evolution of intrinsic drug resistance in the tumors. ROS also are inducers for the expression of various antioxidant enzymes that feed back to suppress ROS levels.

suggested to induce cancer (119). Recent studies linked the roles of inflammation and cancer. In mouse models, suppression of NF-κB signals by induction of IκB superrepressor expression reduced liver cancer progression and prevented the development of HCC in an established HCC model (the *mdr2*-knockout mouse). Reactivation of NF-κB leads to rapid tumor development (197). Moreover, deleting IKKβ in the intestinal epithelial cells reduced colitis-associated tumor incidence resulting from increased epithelial apoptosis during tumor development (82). However, animals with targeted deletion of IKKβ in myeloid cells are more susceptible to endotoxin because of elevated expression of pro-IL-1β. Similar results also were observed by using IKKβ inhibitor ML120B by oral administration. Likewise, Luedde *et al*. (164) observed that ablation of IKKγ in liver parenchymal cells increased the expression of proinflammatory cytokines and chemokines, resulting in the induction of steatohepatitis and spontaneous HCC. These animals showed a striking improvement with a diet supplemented with the antioxidant butylated hydroxyanisole. These results strongly suggest that increased oxidative stress elicited by the ablation of NF-κB signaling is responsible for the proinflammatory response. Collectively, these results further suggest that NF-κB can function as either a cancer-promoting transcription factor or a cancer suppressor, depending on the cancer model.

2. The PI3 kinase pathway. The PI3K pathway is frequently activated by genomic mutations in many types of human cancers (37). Its effects include elevated expression of its subunit (p110), enhanced activities of its downstream effector (Akt), mutations of its suppressor protein phosphatase and tensin homolog deleted on chromosome 10 (PTEN), or a combination of these. The PI3K pathway can be activated by a wide variety of extracellular influences such as growth factors, carcinogens, and hepatitis viral infection (256), and many of these agents are inducers of multidrug transporter gene expression. In prostate cancer cells, it was found that PTEN-negative cells exhibited elevated resistance to doxorubicin and paclitaxel. Inhibition of PI3K with LY29402 potentiates the sensitivity to these antitumor agents (144). It has also been demonstrated that treatment of multidrug-resistant colon cancer cells with the PI3K-inhibitor LY294002 sensitized cells to doxorubicin treatment (144).

It has been demonstrated that PI3K/Akt signaling regulates c-*Myc* promoter activity, mRNA stability, and protein stability through its downstream genes, *GSK3β* and *β-catenin* (132). Furthermore, Akt phosphorylates and sequesters transcription factors FOXO3a, Mad-1, and Miz-1, which inhibit transactivations of c-*Myc* target genes (293). These observations suggest that this signaling pathway can regulate c-*Myc* positively and negatively. PI3K/Akt signaling also regulates the expression of HIF-1α (102). Both c-*Myc* and HIF-1α are regulators of oncogenesis as well as the expression of multiple multidrug-resistance efflux pumps, as described earlier.

3. The p53 pathway. The p53 pathway is one of the major tumor-suppressive mechanisms in mammals. Aberrations in p53 signaling, due to either mutations of p53 or dysfunction of its interacting proteins (MDM-2, MDM-X, P14/P19 ARF), have been found in ~50% of human malignancies (154). The p53 pathway can be activated by a wide variety of external and internal signals, including DNA damage by UV or γ-irradiation, hypoxia, therapeutics, nutritional deprivation, heat shock, oncogenic activation, *etc*. Many of these assaults generate ROS, important regulators of the p53 pathway that controls the expression of hundreds of targeting genes (154, 217). At least two redox-active proteins, redox factor-1 and thioredoxin reductase, have been suggested to regulate the basal level activity of p53. The former functions as a positive regulator by stabilizing (and therefore enhancing) DNA-binding activity of p53 (72), whereas the latter functions as a negative regulator (89, 205, 230). The stability and activity of p53 are markedly enhanced by post-translational modifications such as phosphorylation by the ataxia–telangiectasia mutated (ATM) protein, methylation by methylase, and acetylation by acetyltransferase [for review, see ref. (156)]. Moreover, p53 is subjected to ubiquitination by ubiquitin ligase conferred by MDM-2 protein, which leads to degradation, with a half-life of 6–10 min. By itself, p53 is redox active because it contains several Cys residues at the DNA-binding domains. These Cys residues are important for DNA binding and *S*-glutathionylation by GSH reduces the DNA-binding activity (267).

The role of p53 in drug resistance has been explored in *in vitro* cell-culture studies and *in vivo* systems. Because the p53 signaling pathway can be activated by the therapeutics, one would anticipate that p53 may play a role in drug resistance. Early *in vitro* study demonstrated that wild-type p53 suppressed the expression of human *MDR1* and *MRP1* (229). However, the effects of p53 expression on cancer chemotherapy are complex (72, 272). Clinical investigations showed a correlation between p53 mutations and resistance to cancer chemotherapy in some cancer types, whereas in other tumor types, p53 mutations were correlated with drug sensitivity (72, 272). Given the complexity of the p53 pathophysiologic network in apoptosis, senescence, growth arrest, and DNA damage response, *etc*., it is not surprising that the role of p53 in cancer chemotherapy is very complex.

VIII. Clinical Significance of Redox Signaling in Multidrug Resistance and Therapeutic Opportunities

Since the identification of Pgp as the determinant for the multidrug resistance was established in cultured cells, the importance of its roles in clinical drug resistance has become apparent. Accurate assessment of the role of multidrug transporters in clinical settings requires reliable methods of quantification of the expression levels of these transporters. The expression levels of *MDR1/Pgp1* in clinical specimens have mostly been documented by assaying mRNA levels with real-time polymerase chain reaction (RT-PCR) or by immunohistochemistry for protein levels or both. Both assay systems have inherent weaknesses, including contamination of normal tissues in the tumor specimens (in RNA measurement), and poor sensitivity and specificity (cross-reactivity) and quantifiability (in immunohistochemistry). Moreover, a caveat indicates that levels of expression may not faithfully reflect the functionality of the transporters. For a functional ABC transporter in a clinical setting, at least two criteria should be met: (a) its expression levels should be inversely correlated with the chemosensitivity of antitumor

agents that are known to be substrates of the transporter; and (b) an enhanced response to chemotherapy should be observed when inhibitors or reversal agents are used. The availability of tumor specimens often compromises the ability to perform a thorough study. Given these difficulties, it is not surprising that in-depth studies of the role of the *MDR* transporter in clinical drug resistance is needed, particularly in the areas of solid tumors (149).

Despite this, the expression of *MDR1/Pgp1* has been studied in a wide variety of cancers, including leukemia, myeloma, breast cancer, lung cancer, ovarian cancer, sarcoma, bladder cancer, and cervical cancer (149). We can still correlate cancers that are intrinsically resistant to chemotherapy, such as HCC, renal cell carcinomas, and colorectal cancers with elevated levels of *MDR1* expression (71), although it should be noted that high levels of *MDR1* expression are present in the original neoplastic tissues. Many studies have tried to determine the correlation between expression levels of *MDR1/Pgp1* in these tumors and responses to chemotherapy with antitumor agents that are substrates of *MDR1/Pgp1*. Some studies showed positive correlations between reduced *MDR1/Pgp1* expression levels and improved response rates in some tumor types, whereas other tumor types failed to find such a correlation (134, 149). Expression of the *MRP* family and *ABCG2* in leukemia and in a wide spectrum of solid tumors has been reported [see review in ref. (214)], and similarly observations have been recorded. Among these various tumor types, leukemias have been extensively studied and yielded conflicting results (265). Elevated expression of *MDR1/Pgp1* in adult AML has been shown to correlate with reduced response rates (30, 198, 276, 295). However, in a phase III randomized study of AML patients with inclusion of the *MDR1* inhibitor, valspodar (PSC833), in the mitoxantrone, etoposide, and cytarabine treatment protocol did not improve complete response and overall survival (81). The impacts of *MDR1/Pgp1* (36), the *MRP* family (200, 244), and *BCCRP* (214) in the treatment outcomes of these diseases remain to be conclusively established.

Several approaches have been undertaken to combat multidrug resistance. Conventional approaches include the redesigning of antitumor drugs in such a way that the new agents can escape being eliminated by multidrug transporters. Natural antitumor agents with multiple rings and hydrophobic properties generally are good substrates for these transporters. Therefore, drug design by decreasing the hydrophobicity of the original compound that may reduce the affinity of new agents with Pgp has been rationalized (34). However, this approach so far has proven difficult. Unlike the conventional substrate–enzyme interactions, effecting a small change in a drug structure is likely difficult to produce a substantial reduction in its affinity for the transporter. This is because the drug-binding sites in these multidrug transporters are large and flexible (95). Moreover, merely reducing lipophobicity in drug design may not be sufficient for a successful solution when the complex mechanism of *Pgp1*-mediated drug transport as a whole is considered. It is most important that the parameters that affect the kinetics of drug influx and efflux across the cell membrane ultimately contribute to the intracellular drug accumulation. For example, anthracycline analogues have been designed to circumvent *Pgp1*, thereby enhancing therapy efficacy for treating *MDR* cells. Four anthracycline derivatives, doxorubicin, daunorubicin, 8-(*S*)-fluoroidarubicin, and idarubicin are extruded by Pgp at comparable rates, perhaps because of the presence of an amino sugar group in these four drugs. However, these four drugs have considerable variations in the kinetics of uptake of the drugs, as reflected in differences in cell killing (167).

Early studies in cultured cells showed that the transporter activity of *Pgp1* could be inhibited by verapamil and cyclosporin *A* stimulated considerable interest in clinical trials (189). However, these first-generation Pgp1 reversal agents had only limited success because of toxicity due to pharmacokinetic interactions between the chemotherapeutic agents and the reversal agents. Subsequent clinical trials using the second and third generations of Pgp inhibitors, including tarquidar (XR9576) (164), valspodar (PSC833) (19), zosuquidar (LY335979) (143), ONT-093 (OC-144-093) (49), and elacridar (GF120918) (199), have not generated promising results and require further evaluations. These approaches generally have multiple difficulties, including achieving sufficiently high doses of nontoxic MDR-reversal agents in the clinical setting, the inability to predict that suppression of Pgp activity occurs at such high levels, and the mechanistic importance of Pgp expression in the tumors, as discussed earlier.

Is redox signaling a viable target for overcoming drug resistance in cancer chemotherapy? Recent studies have demonstrated that natural products with antioxidant capacities, such as tea polyphenol and epigallocatechin galate, exhibited reversal effects of multidrug resistance in KB-A-1 cells by 5.2- and 2.5-fold, respectively, but not in drug-sensitive parental cells (172). Treatment of drug-resistant cells with these agents also reduced intracellular ROS levels. These observations suggest that inhibition of MDR in KB-A-1 cells may be attributed in part to the suppression of ROS. Another antioxidant, curcumin, which is a polyphenol, has been shown to be an inhibitor of *MDR1*, *MRP1*, and *BCRP*. Nontoxic concentration of curcumin sensitized the *BCRP*-expressing cells to mitoxantrone, topotecan, SN-38, and doxorubicin. Curcumin apparently is not a substrate of these ABC transporters, but its inhibition may be through stimulating *BCRP*-mediated ATP hydrolysis. Photoaffinity-labeling experiments showed that curcumin also inhibits substrate binding to the *BCRP* transporter (46). Likewise, natural diterpenes, triterpenes, and carotenoids isolated from vegetables and medicinal plants have been shown to enhance substrate accumulation in MDR cells (180). Although the clinical benefits of these antioxidants remain to be demonstrated, they may offer a broad spectrum of modulators in anti-MDR chemotherapy.

Several redox-relevant transduction pathways have been shown to regulate multidrug-resistance transporter gene expression. Therefore, modulation of these specific pathways may be of value for controlling drug sensitivity. Several NF-κB inhibitors have been developed (266). The proteasome inhibitor, bortezomib (velcade, PS-341), has been approved for the treatment of AML, multiple myeloma, and other solid tumors by virtue of its ability to target aberrantly activated NF-κB in these tumors. However, bortezomib is a substrate of Pgp that may diminish its effectiveness in the combination therapy for cancer overexpressing Pgp (176, 218). The PI3K/AKT pathway is implicated in the pathogenesis of

many cancers and is involved in the redox regulation of the multidrug resistance gene. Many inhibitors for this pathway have been under clinical trials, and others are under development (37). LY294002, a specific inhibitor of PI3K/AKT kinase, was found to sensitize vincrinstine-resistant leukemia cells (18).

HIF-1 has been implicated in human cancer development by activating a whole host of genes that are involved in angiogenesis, oncogenic transformation, glucose metabolism, invasion stem cell physiology, and upregulation of the ABC drug transporter (Fig. 6) (234, 235). Overexpression of HIF-1α, perhaps due to intratumoral hypoxia, has been frequently observed in patients of several tumor types [for review, see ref.(202)]. This makes HIF-1α an attractive target of cancer therapy (202). Many antitumor agents have been shown to modulate the activities of HIF-1α by various mechanisms, including decreased HIF-1 protein expression levels, decreased interacting with its DNA binding, and therefore its transactivation of gene expression (233). It is of interest to note that inhibitors to HIF-1α suppressing redox signaling have been in clinical trails for antitumor efficacy (273).

Because of the complexity underlying the mechanisms of carcinogenesis and the emergence of drug resistance in the course of cancer chemotherapy, combination therapy using multiple agents simultaneously targeting multiple pathways has proven to be more effective than that with single agents. Many hundreds of genes are regulated by each of these individual pathways, but only a subset of genes is relevant to cancer progression and drug resistance. Moreover, the contributions of these signal-transduction pathways may vary among different types of cancer. Thus, identifying an effective treatment protocol will be a long process, despite the vast amounts of information that have been generated from preclinical investigations.

IX. Redox Signaling in Multidrug-Resistant Cancer Stem Cells

The concept of cancer-initiating cells was originally developed from the work in human AML. Lapidot *et al*. (142) identified a rare population of $CD34^+CD38^-$ cells from patients with AML, which, when infused into severe combined immune-deficient mice, resulted in leukemic blast generation, whereas similar infusions with more committed $CD34^+CD38^+$ lineage cells or $CD34^-$ cells failed to produce leukemia. $CD34^+CD38^-$ cells maintain the differentiating and proliferative capacity with potential self-renewal properties in nonobese diabetic mice with severe combined immunodeficiency disease (29). Hematopoietic stem cells (HSCs) remain the best-characterized system.

A. Multidrug-resistant hemopoietic stem cells

Evidence indicating that ABC transporters play a critical role in protecting hematopoietic stems cells was originally described by Chaudhary and Ronninson (45). These investigators showed that populations of bone marrow cells exhibiting reduced retention of fluorescent rhodomin (Rho 123) and elevated expression of *MDR1* are associated with hematopoietic progenitor ($CD34^+$) cells. This population of cells, commonly referred to as side population (SP), can be isolated by fluorescence-activated cell sorting (FACS) by the virtue of their enhanced ability to efflux fluorescent dyes such as Rho123 and Hoechst 33342 (78). ABCG2 was subsequently identified and characterized as a novel stem cell marker (292). Interestingly, ABCG2-derived SP cells were also found within embryonic stem (ES) cells, as well as many solid tissues. By using knockout strategies, it was demonstrated that no significant hematologic defects were associated with *mdr1a*$(-/-)$*mdr1b*$(-/-)$ compound mice (225), and a normal population of SP cells was observed in the bone marrow, suggesting that other ABC transporters may play a compensatory role in the stem cell population (39, 292). However, ABCG2$(-/-)$ animals showed a reduced SP population and were significantly more sensitive to mitoxantrone in drug-treated transplanted mice (291), demonstrating that ABCG2 constitutes the major SP cells and is associated with drug resistance. It should be noted that, strictly speaking, the SP isolated by FACS may not be necessarily enriched in ABC transporters, because reduced fluorescence intensity is not always correlated with elevated expression levels of efflux pumps. Many factors can affect the fluorescence intensity, including reduced rates of dye uptake, reduced stability of the fluorochromes, enhanced quenching of fluorescence due to intracellular sequestration of the fluorochromes and their inteactions with cellular constituents and organelles, etc.

HSCs reside in bone marrow where oxygen supplies are low and are sensitive to oxidative stress. Loss of the mitochondrial antioxidative stress enzyme SOD2 in HSCs enhanced protein oxidative damage and resulted in erythroid hypoplasia (74). Interestingly, maintenance of the self-renewal capacity of HSCs requires a functional ataxia–telangiectasia mutated (*ATM*) gene. *Atm*$(-/-)$ mice showed drastically reduced HSC numbers that are associated with elevated ROS production. These phenotypes are reversed by using the membrane-permeable antioxidant, NAC (110). Further study revealed that ROS-induced defects in HSC maintenance were due to p38 MAPK phosphorylation (111). These results demonstrated that ROS play an important role in the regulation of HSC homeostasis.

Elevated expression of BCRP is present in SP cells derived from a wide range of tissues, including muscle, heart, lung, intestine, and cornea. Perturbation of *BCRP* expression affects the SP cell phenotype. Downregulation of ABCG2 by siRNA depletes retinal SP cells and promotes their differentiation (24). These results correlate the expression of *BCRP* expression with the maintenance of SP cells.

B. Multidrug-resistant cancer stem cells of solid tumors

Cumulative evidence has demonstrated that SP cells are present in a wide variety of solid tumors, as well as their derived cell lines (58, 97), including lung cancer cell lines and human lung cancer samples (97), hepatocellular carcinoma (50), breast cancer (6, 241) mesenchymal tumors [osteosarcomas, chondrosarcomas, leiomyosarcomas, cordomas (275)], gastroinstestinal cancer cells (90), murine ovarian cancer (246), *etc*. In some studies, when *ABCG2* expression levels were analyzed, SP cells displayed elevated expression of *ABCG2* as well as other ATP-binding cassette transporters. The *ABCG2* and *ABCB1/MDR1* genes are expressed in normal stem cells and in most tumor stem cells (125, 222, 292). *ABCG2* levels were reduced when stem cells were induced to differentiate. Levels of *ABCG2* decreased as MCF-7 SP cells

were treated with TGF-β which induces epithelial–mesenchymal transition (285).

Recent studies have identified an additional ABC transporter, ABCB5 (124, 223), as a marker for human melanoma stem cells. ABCB5 was originally characterized as a determinant of membrane potential that controls cell fusion in skin progenitor cells. ABCB5 functions as a Rho 123 efflux pump and a major efflux mediator of doxorubicin and contributes to chemoresistance to doxorubicin (73). ABCB5 marks CD133-expressing progenitor cells, a surface marker protein that is present in the stem cell population of many solid tumors, including glioma (17), colon cancer (190), and prostate cancer (54). ABCB5 appears to be important for the growth of melanoma in animal xenografts, because administration of monoclonal antibody directed at ABCB5 suppresses tumor growth (223), providing a potential strategy for targeting cancer stem cells by using the anti-ABC transporter approach. However, it remains to be investigated whether ABCB5 is the major efflux transporter that contributes to the inherent resistance of melanomas to many chemotherapeutic interventions.

C. Signaling pathways in cancer stem cells

In addition to the efflux multidrug transporters, several crucial signaling pathways have been elucidated for cancer stem cell biology, including Wnt/β-catenin, Notch, sonic hedgehog, Bmi-1, the Hox family, and PTEN (161). Here, only the pathways that are most relevant to multidrug resistance are discussed.

1. The Wnt/β-catenin signaling. The Wnt signal-transduction pathway is a well-conserved signaling process through evolutionary processes. It plays an important role in regulating cellular proliferation, differentiation, motility, and survival/death (248). Wnt proteins are 39- to 46-kDa secretory glycoproteins that bind the cell-surface receptors, Frizzleds (Fig. 15B). The Wnt signal is frequently activated in various human cancers (175). Activation of Wnt signaling prevents cytoplasmic β-catenin from degradation, allowing it to translocate into the nucleus, where it binds and activates transcriptional repressor T-cell factors (TCFs), resulting in upregulation of the target genes, one of which is human MDR1. The promoter of *MDR1* contains a cluster of TCF/β-catenin–responsive elements through which expression of *MDR1* by this transcriptional machinery is regulated (281). One of the intracellular repressors is adenomatous polyposis coli (APC). Deletions of *Apc* alleles result in the development of intestinal polyps. Interestingly, this group of investigators found that expression of murine *mdr1a/mdr1b* suppressed the development of colonic polyps in *APC* $^{min/+}$ mice (280), suggesting that *mdr1a/mdr1b* may function as a tumor suppressor in this tumor model.

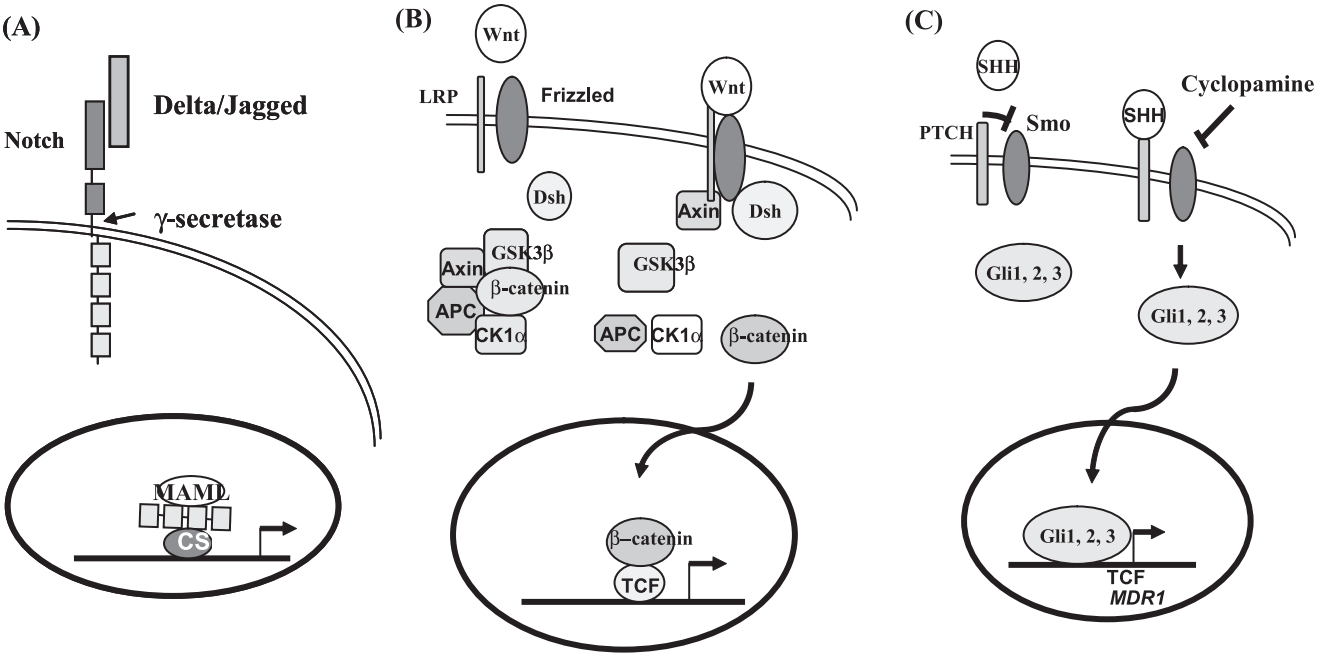

FIG. 15. The Notch, Wnt, and SHH signaling pathways. (**A**) Notch pathway: Ligands (Delta or Jagged) binding to the receptor trigger proteolytic cleavage by γ-secretase, resulting in release of intracellular domain of the receptor (NICO), which subsequently forms a complex with the transcription factor CSL and cofactor Mastermind-like (MAML) family to activate target-gene expression. (**B**) Wnt pathway: In the absence of Wnt ligand, β-catenin is complexed with a group of proteins, including adenomatous polyposis coli (APC) and Axin, where it is phosphorylated by casein-kinase 1a (CK1a) and glycogen synthase kinase 3β (GSK3β). On activation by the binding of Wnt ligand to the Frizzled and lipoprotein-related protein (LRP) receptors, Frizzled then binds to disheveled (Dsh), resulting in LRP phosphorylation, and Axin is translocated to the cell membrane. β-Catenin is accumulated and translocated to the nucleus and interacts with the T-cell factor (TCF) transcription factor (adapted from ref. 7). (**C**) SHH pathway: the receptor of SHH is the tumor-suppressor gene product, PTCH, which represses the activation of smoothened (Smo). Binding of SHH releases this repressive effect on Smo by PTCH, activating glioblastoma transcription factor (Gli1, 2, or 3 family), which than activates the transcription of target genes.

The involvement of Wnt/β-catenin signaling in redox regulation can be found from the following studies. β-Catenin is a cofactor that activates FoxO transcription factors, which are regulated by PI3K/Akt oxidative-stress signaling (69), the same stress signaling that regulates the expression of many *MDR* transporters. Moreover, members of FoxO have been shown to protect HSCs from oxidative stress. Conditional triple deletion of *FoxO1*, *FoxO3*, and *FoxO4* in the adult hematopoietic cells resulted in marked increase in ROS levels with defective myeloid-lineage expansion and long-term repopulating of HSCs. Treatment with the˘antioxidative NCS reversed the observed phenotype in HSCs (260), further supporting the role of ROS in the maintenance of the HSC population.

2. The Notch signaling. Notch was discovered as an oncogene in humans that gives rise to T-cell leukemia, bearing the t(7,9)(q34,q34) translocation (68). The Notch signaling pathway is well preserved from nematodes to humans. Four Notch receptors exist for four Notch ligands. On binding of Notch ligands (Delta or Jagged) to the receptors, proteolytic cleavages by γ-secretase liberates the Notch intracellular domain (NCID), which then translocates into the nucleus, and transactivates the target genes by complexing with the transcription factor CSL and cofactors of the Mastermind-like (MAML) (Fig. 15A). Recent studies demonstrated that the Notch signaling pathway plays important roles in modulating cell-fate decisions, differentiation, survival/apoptosis, and cell-cycle control. Importantly, Notch signaling has an important function in stem cell biology. Manipulating the expression of Notch ligand or its downstream target affects the self-renewal capacity of HSCs (27).

The Notch signaling is also redox regulated. Early study demonstrated that mutations in Notch were associated with mitochondrial dysfunction (57). More recent study demonstrated that disruption of Notch signaling by γ-secretase inhibitors resulted in the accumulation of ROS (25). Furthermore, constitutive activation of the Notch signal by overexpressing NCID results in the upregulation of ABCG2, whereas cells treated with Notch signal inhibitor reduced the ABCG2 population, suggesting that Notch signaling regulates ABCG2 and SP populations (24). It remains to be determined whether upregulation of ABCG2 by Notch signaling involves oxidative stress.

3. The sonic hedgehog signaling. The sonic hedgehog (SHH) signaling pathway performs various important functions, which include neural tube patterning, left–right symmetry, polarizing activity in the limb, and morphogenesis of various organs (8). It also plays an important role in the development of various cancers. The receptor for SHH is the product of the tumor-suppressor gene, patched transmembrane receptor (*PTCH*). In the absence of SHH ligands, *PTCH* activates smoothened (Smo) membrane protein. Activation of SHH signals elicited by SHH binding to *PTCH* relieves the repression of Smo, thereby activating glioblastoma transcription factors Gli1, Gli2, and Gli3, and turning on the expression of the targeting genes (Fig. 15C). Abnormalities in the SHH signaling pathway, including mutations of *PTCH2*, *Smo*, *Gli1*, and *Gli2*, contribute to the development of malignancies in humans (44).

A recent study demonstrated that inhibiting SHH signaling by cytopamine enhanced resistance to docetaxel, methotrexate, and etoposide by upregulation of the expression of Gli-1, resulting from activations of *MDR1* and *BCRP* expression. Downregulation of *Gli* expression by the siRNA approach reduced the expression levels of *MDR1* and *BCRP*. These results demonstrate that SHH signaling regulates the expression of these ABC transporters (239) and supports their dual roles in drug-resistance gene regulation and stem cell maintenance.

X. Therapeutic Opportunities for Cancer Stem Cells

Cancer stem cells share many properties with those found in leukemia-initiating cells. They are rare in numbers (10^{-3}–10^{-5} % of all cancer cells) and normally reside in an environment where O_2 tension is low. They are generally replicatively quiescent. Some cancer stem cells overexpress multidrug transporters, but this is not the rule. They may be less sensitive to oxidative stress exerted by cytotoxic agents, but redox signals play an important role in the maintenance of their "stemness." They exhibit highly proliferative activity in xenotransplantation in adaptive and innate immunity-deficient animals. In some cases, a few hundred cells can initiate tumors in these animals (212).

It is thought that traditional treatments such as chemotherapy and radiation therapy do not readily eradicate cancer stem cells because of their intrinsic properties and because of their microenvironmental niche (Fig. 13). This raises an important question of whether cancer stem cells represent a drug-resistant population that contributes to therapeutic failure in initial treatment. Supporting evidence has been suggested by a recent finding that in the $CD34^+CD38^-$ leukemic cells candidate HSC subpopulation, enrichment of *MDR1* and/or *BCRP* mRNA levels were found in eight of 10 AML patients that were nonresponsive to daunorubicin plus cytarabine treatment; whereas it was found in none of seven patients with complete remission. No such association was found in more-differentiated $CD34^+CD38^-$ (96). These results suggest that expression of *MDR1* and *BCRP* in leukemic stem cells plays a role in the chemotherapy response in AML patients. Another support to this hypothesis may be the finding that radiation therapy for glioma resulted in an enriched stem cell population that is resistant to subsequent ionizing radiation by activating a DNA-damaging response ATM signaling (17).

Because HSCs can be isolated and engrafted back into the host, a strategy was developed for ex vivo gene therapy by using retroviral *MDR1* vector-transduced HSCs to protect the bone marrow from toxicity after high-dose chemotherapy. Three clinical protocols (88, 94, 181) have been conducted involving in HSCs collected from patients with breast cancers. In these protocols, a replication-incompetent retrovirus containing the *MDR1* gene in the presence of stem-cell factors was *ex vivo* transduction into the HSC population, followed by autologous bone marrow transplantation [for review, see ref. (60)]. The results are disappointing because of low *ex vivo* transduction efficiency in combination with low levels of *MDR1* expression. These studies have several caveats. Transduction of murine bone marrow cells with a functional *MDR1* retroviral vector resulted in an almost 2-log increases in SP cell numbers, whereas functionally inac-

tive MDR1 mutant cDNA was unable to amplify phenotypically defined SP cells and functionally defined repopulating cells. Another caveat is that forced expression of *MDR1* in murine bone marrow cells resulted in the development of an adverse myeloproliferative syndrome in transplanted mice, characterized by high peripheral white blood cell counts due to myeloid progenitor accumulation. It was also found that the role of MDR1 protein in transplanted bone marrow did not provide a benefit in the treatment of chronic myelocytic leukemia model (CML) with imatinib (296). Moreover, the amount of ABCG2 expression in the myeloid leukemia cells (AML) was very low to reach the levels that are comparable for drug-resistance levels *in vitro* (1). However, it is important to note that no detectable *ex vivo* expansion of the SP population was found when the *MDR1* transduction experiments were carried out in a nonhuman primate model, perhaps resulting from the differences in transduction efficiencies between the mouse and rhesus monkey (232). Future efforts should be focused on technical aspects by improving the transduction efficacy as well as levels of drug-resistance gene expression by using a strong promoter in the recombinant vectors. The potential adverse effects of overexpressed MDR product in the repopulated bone marrow also should be investigated.

Despite these disappointing results, HSCs may still have value for cell and gene therapy in the multidrug-resistance arena. It is conceivable that the maintenance of HSCs is a balance of self-renewal *versus* differentiation. Because of current knowledge of novel methods to expand stem cell self-renewal, one may generate a sufficient quantity of HSCs for transplantation into patients with marrow depletion due to chemotherapy (15, 29).

Several additional approaches can be considered for eradicating cancer stem cells. First, because many cancer stem cells overexpress *MDR1* and/or *BCRP*, the incorporation of inhibitors of ABC transporters into the chemotherapeutic regimens may be of value. For example, pheophorbide, a chlorophyll catabolite, is a highly specific inhibitor of *ABCG2* (215). Second, because cancer stem cells maintain both self-renewal and differentiative ability, induction of differentiation into a committed cell lineage may abrogate their proliferative capacity. Many differentiation-inducing agents have been developed for the lineage studies of embryonic stem cells. Some of these inducers may be of value for this purpose (76). Third, many cancer stem cells exhibit elevated signaling-transduction pathways including Notch, Wnt, and SHH pathways. These signaling pathways are regulated by oxidative stress. Inhibitors for these pathways are being developed (215).

As many multidrug-resistance efflux pumps are membrane proteins, monoclonal antibodies tagged with antitumor agents against the extracellular domains of these membrane proteins may be of important value for the treatments of efflux pump–overproducing cancer stem cells. This approach may be particularly attractive, considering that cancer stem cells are rare inside the tumor mass so that high specificity of targeting is in demand. The successful use of a monoclonal antibody against ABCB5 in melanoma stem cells has at least proven the principle (223).

Because cancer stem cells share many common features with those found in normal stem cells, caution must be exercised to avoid harming the normal stem cells (such as those in the bone marrow) during treatment. As better understanding of the role of cancer stem cells in the evolution of drug resistance is reached, one can expect that better treatment modalities targeting cancer stem cells also will be developed.

XI. Conclusions: Challenges and Perspectives

Almost three decades have passed since Pgp1 was first identified as a multidrug-resistance transporter. The field has generated enormous research enthusiasm in the hope that mechanistic insights can be translated into therapeutic benefit and that one of the most important problems in cancer chemotherapy—multidrug resistance—will be solved. It has been learned that multidrug resistance is much more complex than originally conceived. First, much redundancy exists in multidrug-resistance transporter genes in our genome. Each of these transporters not only displays overlapping substrate specificity but also is frequently co-expressed in many normal tissues and tumors. Second, the activities of these multidrug transporters can be upregulated by many extracellular influences. Some of the upregulation mechanisms are somewhat specific to particular drug transporters, but many are general and can affect many physiologic pathways. Third, compensatory mechanisms affect the expression of these transporters. We previously demonstrated that animals lacking *mdr1a* by knockout strategy upregulate *mdr1b* expression (16). Fourth, perhaps the most difficult task is delineating the unknown mechanisms associated with multidrug resistance evolved during the course of chemotherapy. Emergence of drug resistance is the result of a dynamic battle between cancer cells and chemotherapeutic agents. Drug-resistance mechanisms acquired during chemotherapy can be multifactorial, not only by the escape from the primary targets to which the chemotherapeutic agents act on, but also by the evolution of new mechanisms due to second hits. These new mechanisms can be due to genetic or epigenetic alternations, cancer cells and microenvironment interactions resulting in alterations of pharmacokinetic and pharmacodynamic properties of cancer drugs, and accessibility to the available drug delivery systems, *etc*. Although new antitumor agents will continue to be developed, it is anticipated that novel drug-resistance mechanisms will follow. Because of the versatility of redox signaling that could virtually reach almost every part of a cell and affect many signaling processes, it is anticipated that redox signaling will continue to play an important role in regulation of drug resistance with new therapeutics.

The challenges that face drug resistance in cancer chemotherapy are formidable and require that we translate the growing body of knowledge gained from drug-resistance research into effective therapeutic measures. Over a period of many years, evidence has accumulated suggesting that redox signaling is intimately associated with tumor progression. The enormous amount of information that has been generating from laboratory and preclinical investigations has revealed that redox signaling is a major stress-response process associated with the treatment of antitumor agents and is an important regulator of the expression and activity of many multidrug-resistance transporters.

The concept of drug-resistant stem cells in cancer chemotherapy has been around for many years. Recent studies

showing that elevated expression of multidrug-resistance efflux pumps (*e.g.*, MDR/Pgp1, BCRP, and ABCB5) are associated with cancer stem cells demonstrate that drug-resistant stem cells and cancer stem cells share some common intrinsic properties, at least in some tumor types. These observations have brought together the two important fields of cancer research. Aside from the regulation of these ABC transporters, the important roles of redox signaling in controlling the "stemness" of these cancers are highlighted here.

Thus, strategies aimed at modulating cellular response with the use of redox modulators should be promising, because these strategies, in principle, provide a dual effect on cancer treatment: one is the cytostatic effect of tumor growth, and the other is the modulation of drug resistance in cancer chemotherapy. Nonetheless, one may have to bear in mind that whereas redox-targeting strategies may be promising, by themselves, may not be sufficiently effective to eradicate tumor cells, especially when the tumor burden is high. In this regard, integrating redox modulators into conventional chemotherapy, or other means of therapeutic interventions, may improve the overall treatment efficacy. All in all, the ultimate benefits of these strategies remain to be clinically documented.

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Abbreviations

2-AAF, 2-acetylaminofluorene; ABC, ATP-binding cassette; ABR, abnormal banding regions; Akt, protein kinase B; d-ALA, D-aminolevulinic acid; AML, acute myeloid leukemia; APAP, acetaminophen; APC, adenomatous polyposis coli; ARE, antioxidant response element; ASK1, apoptosis signal-regulating kinase 1; *ATM*, ataxia telangiectasia mutated gene; BCRP, breast cancer resistance protein; B-F-B, break-fusion-bridge; *BRCA1*, breast cancer 1; BSO, buthionine sulfoximine; CK1α, casein-kinase 1α; CHO, Chinese hamster ovary; CK1, casein kinase 1; CML, chronic myelocytic leukemia; COX1, cyclooxygenase 1; COX2, cyclooxygenase 2; Cul3, Cullin 3; Cys, cysteine; CYP450, cytochrome P450; DM, double minute; DNMTs, DNA methyltransferases; NA-PK, DNA protein kinase; DNR, daunorubicin; DEN, diethylnitrosamine; DSB, double-strand break; Dsh, Dishevelled; EBV, Epstein–Barr virus; ER, endoplasmic reticulum; Ero1, ER oxidoreductin; ES, embryonic stem; FACS, fluorescence-activated cell sorter; FAD, flavin adenine dinucleotide; γ-GCSh, γ-glutamylcysteine synthetase heavy subunit; Gli, glioblastoma transcription factor; Glu, glutamine; Gly, glycine; GSH, reduced glutathione; GSSG, oxidized GSH; GST, glutathione S-transferase; HIF-1, hypoxia-inducible factor-1; HuR, a member of ELAV family AU-rich binding RNA-binding protein; GSK3β, glycogen synthase kinase 3β; HADC, histone deacetylase; HBV, hepatitis virus B; HCC, hepatocellular carcinoma; HPETE, hydroperoxyeicosatetraenoic acid; HSC, hematopoietic stem cell; IκB, inhibitory protein kappa B; IKK, IκB kinase; IL, interleukin; IRAK, interleukin-1 receptor–associated kinase; JNK, NH_2-terminal kinase; LAP, lipoprotein-related protein; LRP, lung resistance–related protein; LTC4, leukotriene C4; MAML, mastermind-like; MAPK-APK2, mitogen-activated protein kinase–activated protein kinase 2; MeCp2, methyl-CpG-binding protein-2; MAPK, mitogene-activated protein kinase; MKK, MAP kinase kinase; MKKK; MAP kinase kinase kinase; MSD, membrane-spanning domain; MA, S-adenosylmethionine synthetase; MAT, methionine adenosyltransferase; MXR, mitoxantrone resistance protein; MDR, multidrug resistance; MRP, multidrug-resistance protein; NAC, *N*-acetylcysteine; NCID, Notch intracellular domain; NHEJ, nonhomologous DNA end-joining; NF-κB, nuclear factor-kappa B; NIK, NF-κB–inducing kinase; NQO, NAD(P)H quinine oxidoreductase; Nrf2, NF-E2–related factor; PDI, disulfide isomerase; Keap1, Kelch-like ECH-associated protein; PDK, phosphoinositide-dependent protein kinase; Pgp, P-glycoprotein; PHD, prolyl hydroxylase-domain protein; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PP, peroxisome proliferator; PPARγ, peroxisome proliferator–activated receptor γ; PTCH, patched transmembrane receptor; PTEN, phosphatase and tensin homologue deleted on chromosome 10; redox, reduction-oxidative; Rho 123, rhodamin 123; ROS, reactive oxygen species; RILP76(RALBP-1), Ral-binding protein 1; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SN-38, 7-ethyl-10-hydroxy-camptothecin; SHH, sonic hedgehog; Sin3, SWI-independent 3; Smo, smoothened; SOD1, superoxide dismutase 1; SP, side population; TCF, T-cell factor; TLR, Toll-like receptor; TMD, transmembrane domain; Trx, thioredoxin; VHL, von Hippel–Lindau.

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