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Modulation of NRF2 signaling pathway by nuclear receptors: Implications for cancer

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ABSTRACT

Nuclear factor-erythroid 2 p45-related factor 2 (NRF2, also known as Nfe2l2) plays a critical role in regulating cellular defense against electrophilic and oxidative stress by activating the expression of an array of antioxidant response element-dependent genes. On one hand, NRF2 activators have been used in clinical trials for cancer prevention and the treatment of diseases associated with oxidative stress; on the other hand, constitutive activation of NRF2 in many types of tumors contributes to the survival and growth of cancer cells, as well as resistance to anticancer therapy. In this review, we provide an overview of the NRF2 signaling pathway and discuss its role in carcinogenesis. We also introduce the inhibition of NRF2 by nuclear receptors. Further, we address the biological significance of regulation of the NRF2 signaling pathway by nuclear receptors in health and disease. Finally, we discuss the possible impact of NRF2 inhibition by nuclear receptors on cancer therapy.

1. Introduction

In general, people are exposed to a wide variety of environmental pollutants and toxicants that generate oxidative stress in cells. Such stress elevates the generation of reactive oxygen species (ROS) that cause DNA damage such as base modification (modification of guanine, causing G → T transversions), rearrangement of the DNA sequence, single-strand breaks, and mutations in tumor-suppressor genes that ultimately lead to the initiation, promotion, and progression of cancer [1,2]. ROS are oxygen by-products from exogenous and endogenous sources. Numerous studies suggest that oxidative stress in cells causes not only cancer but also other chronic diseases such as diabetes, arthritis, asthma, chronic obstructive pulmonary disease, and neurodegenerative diseases [3]. Nuclear factor erythroid 2-related factor 2 (NRF2) is a transcription factor which belongs to the Cap 'N' Collar family that contains a highly-conserved basic leucine zipper structure [4]. Under oxidative stress, NRF2 regulates the series of genes known as antioxidant response elements (AREs) or electrophile response elements. ARE and/or electrophile response element genes express various antioxidants and phase-II cytoprotective and detoxifying enzymes such as glutathione S-transferases (GSTs), NAD(P)H:quinone oxidoreductase (NQO1), aldo-keto reductase, and heme oxygenase-1. These enzymes are responsible for the detoxification and neutralization of xenobiotics and ROS, and so protect against the diseases associated with oxidative stress [5,6]. In addition, it has also been shown that NRF2 not only regulates cytoprotective gene expression but also cell proliferation [7].

2. KEAP1–NRF2–ARE signaling pathway

2.1. KEAP1 (Kelch-like ECH-associated protein 1)

KEAP1 is a 69-kD, cysteine-rich protein (27 cysteine residues), a substrate adaptor for cullin(Cul3)-containing E3 ubiquitin ligase. KEAP1 contains five domains (Fig. 1A): an N-terminal region, a BTB dimerization domain (Broad-Complex, Tramtrack, and Bric a′ brac), a cysteine-rich intervening region (IVR domain), a Kelch domain/double glycine repeat (DGR) domain possessing 6 Kelch repeats, and a C-terminal region [8]. The BTB domain is crucial for KEAP1 homodimerization and interaction with the Cul3-based ubiquitin E3 ligase complex for NRF2 ubiquitination [9,10]. A Cys151 residue in the BTB domain plays an

Abbreviations: AKR1C, aldo-keto reductases 1C1 and 1C2; ARE, antioxidant response element; ATRA, all-trans retinoic acid; Bach1, BTB and CNC homologue 1; BBN, (N-nitrosobutyl(4-hydroxybutyl)amine); Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma—extra-large; β-TrCP, β-transducin repeat-containing protein; CBP, CREB binding protein; ChIP, chromatin immunoprecipitation; DBD, DNA-binding domain; Dex, dexamethasone; DPP3, dipeptidyl peptidase III gene; ERα, estrogen receptor alpha; ERRβ, estrogen-related receptor beta; GR, glucocorticoid receptor; GSH, reduced glutathione; GST, glutathione S-transferase; KEAP1, Kelch-like ECH-associated protein 1; miRNA, microRNA; Neh, NRF2–ECH homology; NQO1, NAD(P)H:quinone oxidoreductase 1; NRF2, NF-E2 p45-related factor 2; PPAR, peroxisome-proliferator-activated receptor; ROS, reactive oxygen species; RAR, retinoic acid receptor; RXRα, retinoid X receptor alpha; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; SNP, single-nucleotide polymorphism; TXS, thromboxane synthase; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1

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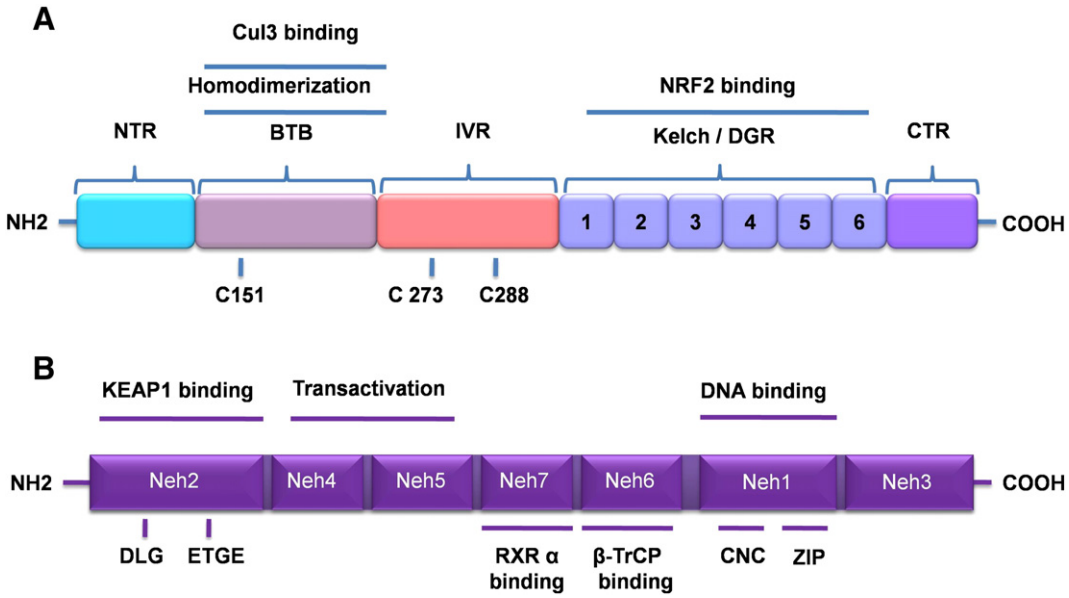

Fig. 1. Domain structures of KEAP1 and NRF2. (A) KEAP1 has five domains: (1) the N-terminal domain, (2) a BTB domain responsible for KEAP1 homodimerization and association with Cul3, (3) IVR, a cysteine-rich domain that acts as a sensor for NRF2 inducers, (4) the Kelch/DGR domain that is important for binding with the Neh2 domain of NRF2, and (5) the C-terminal domain. (B) NRF2 contains seven domains known as Neh1–Neh7. The N-terminal domain Neh2 contains two motifs, DLG and ETGE, which are responsible for binding KEAP1. The Neh3, Neh4, and Neh5 domains are important for the transactivation activity of NRF2. The Neh7 domain binds RXRα and inhibits ARE gene activity. Neh6 is a serine-rich domain required for β-TrCP binding. The C-terminal domain, Neh1, is a leucine zipper motif responsible for DNA-binding and dimerization with Maf.

important role in NRF2 activation [11]. The IVR domain (also known as the BACK domain) is sensitive to oxidation and has critical, highly-reactive cysteine residues such as Cys273, Cys288, and Cys297, that act as sensors for NRF2 inducers [12]. Moreover, Cys288 and Cys297 are critical for the repression of NRF2 activity [13]. The DGR domain contains six repetitive Kelch structures that specifically bind to the conserved N-terminal Neh2 domain of NRF2 and negatively regulate its activity [14]. In addition, Niture et al. showed that the DGR domain of KEAP1 binds with the BH2 domain of Bcl-2 (B-cell lymphoma 2) and targets the Cul3-Rbx1-mediated degradation of Bcl-2 [15]. The DGR and C-terminal region domains are collectively named the DC domain. The BTB-DC domains together play a crucial role in NRF2 proteasomal degradation and repression.

2.2. NRF2

NRF2, the key activator of the pathway, has seven functional domains: Neh1–Neh7 (NRF2–ECH homology) (Fig. 1B). Neh1 contains a basic leucine zipper motif that heterodimerizes with small musculoaponeurotic fibrosarcoma protein, DNA, and other transcription partners [16]. Plafker et al. have shown that the Neh1 domain binds with ubiquitin-conjugating enzyme to regulate the stability and enhance the transcriptional activity of NRF2 [17]. Neh2, the N-terminal domain, contains two motifs known as DLG and ETGE. These motifs are essential for the interaction between NRF2 and the Kelch domains of KEAP1, the stability of NRF2, and NRF2 ubiquitination. However, the DLG motif has less affinity for Kelch domains than the ETGE motif [18,19]. The carboxy-terminal Neh3 domain interacts with the transcription co-activator known as CHD6 (a chromo-ATPase/helicase DNA-binding protein), which is critical for the transactivation of ARE-dependent genes [20]. The Neh4 and Neh5 domains bind with the CH3 domains of another transcriptional co-activator, CBP (CREB-binding protein), which mediates the transactivation of NRF2 target genes [21]. In addition, a recent study has shown that these two transactivation domains interact with the nuclear cofactor RAC3/AIB1/SRC-3 and enhance NRF2-targeted ARE gene expression [22]. Specifically, Neh5 has a redox-sensitive nuclear-export signal that regulates the cellular localization of NRF2 [23]. Neh6 is a serine-rich domain containing two motifs (DSGIS and DSAPGS) that interact with β-transducin repeat-containing protein (β-TrCP) that acts as a substrate receptor for the Skp1–Cul1–Rbx1/Roc1 ubiquitin ligase complex. These two distinct β-TrCP recognition motifs of the Neh6 domain are important to control the stability of NRF2 with regard to glycogen synthase kinase-3-targeted SCF/β-TrCP-dependent degradation. It is noteworthy that Neh6 controls the stability of NRF2 in a KEAP1-independent manner [24–26]. Recently, we discovered a seventh domain that we named Neh7. Neh7 specifically interacts with retinoic X receptor alpha (RXRα), a nuclear receptor that inhibits the NRF2–ARE signaling pathway [27].

2.3. Regulation of NRF2 by KEAP1

Under basal homeostatic conditions, NRF2 is tethered in the cytoplasm by the actin-bound protein KEAP1, also known as INRF2 (inhibitor of NRF2) [8,28] (Fig. 2). KEAP1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex that targets NRF2 for ubiquitylation and subsequent proteasomal degradation. The highly-reactive cysteine residues in KEAP1 are involved in the degradation and the stabilization of NRF2 [29].

Oxidative stress and NRF2 inducers activate NRF2 in cells where it escapes from KEAP1 retention, which leads to enhanced accumulation of NRF2. Thereby, NRF2 translocates into the nucleus where it heterodimerizes with small Maf proteins and transactivates ARE-driven gene expression [30] (Fig. 2). It is important to note that small Maf proteins such as MafF, MafG, and MafK belong to the basic leucine zipper transcription factor family, which plays a crucial role in mammalian gene regulation [31]. Small Maf proteins form heterodimers with members of the CNC transcription factors that bind with ARE, and also form homodimers that bind with specific DNA motifs known as Maf-recognition elements, both acting as transcriptional regulators [32,33]. It has been suggested that NRF2–MafG heterodimerization causes the masking of a nuclear export signal in the NRF2 basic leucine zipper domain that leads to the enhanced retention of NRF2 proteins in the nucleus [34].

The exact molecular mechanism of NRF2 activation by thiol modification remains in question. However, several lines of evidence suggest that the NRF2 inducers form an intermolecular disulfide bond between

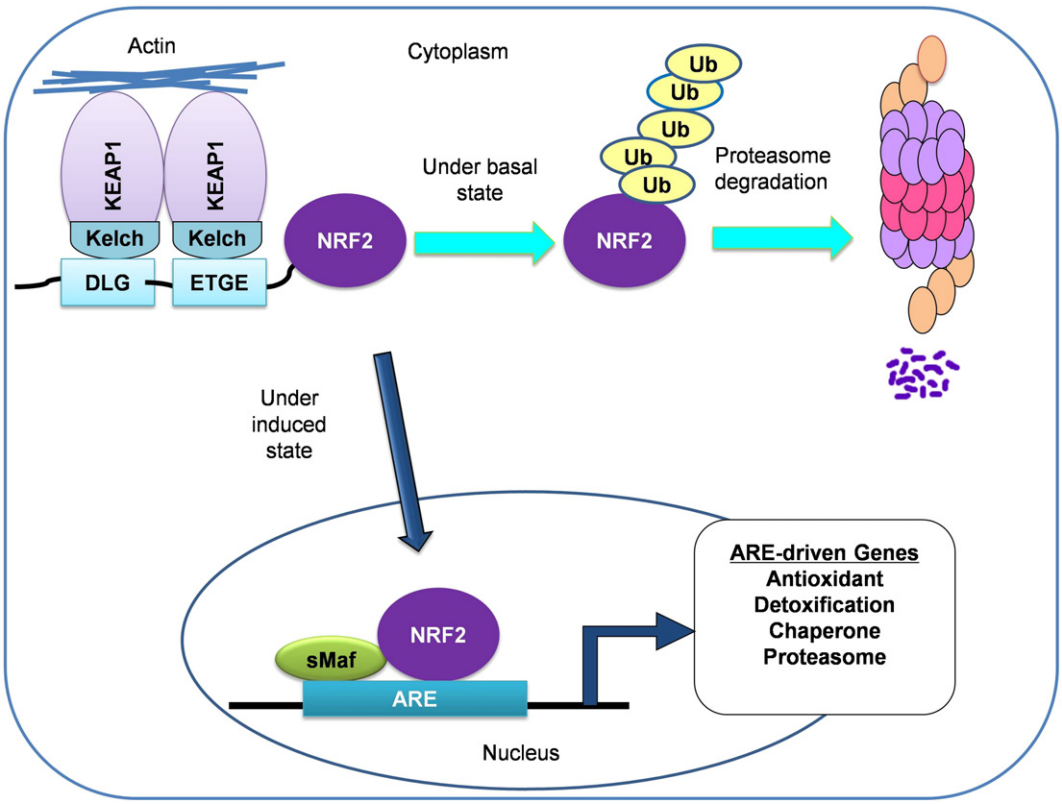

Fig. 2. The KEAP1–NRF2–ARE pathway. In the basal state, NRF2 binds to KEAP1 at its two motifs (ETGE and DLG). KEAP1 ubiquitinates NRF2 and this is followed by proteasomal degradation. In the induced state, NRF2 is protected from KEAP1 repression and translocates into the nucleus. In the nucleus, NRF2 binds to small Maf proteins through the Neh 1 domain which is responsible for the transactivation of ARE gene expression.

Cys273 of one KEAP1 monomer and Cys288 of the second monomer in the KEAP1 dimer. Therefore, formation of the disulfide bond causes the DGR domains in dimeric KEAP1 to separate, leading to the stabilization of NRF2 [13]. Moreover, the NRF2 inducer sulforaphane predominantly causes a conformational change in KEAP1 by forming a thionoacyl adduct in the Kelch domain, rather than in the central linker domain, leading to NRF2 stabilization [35]. Similarly, Fourquet et al. showed that exposure to H_2O_2 and nitrosative agents promotes the formation of a disulfide link between two KEAP1 molecules through the reactive Cys151 residue that leads to the liberation of NRF2 from the KEAP1 dimer [36]. Interestingly, Bensasson et al. showed that diphenols induce NQO1 expression *via* a two-step process: (i) oxidation of diphenol inducers to their quinone derivatives, and (ii) oxidation of highly-reactive thiols of the protein sensor KEAP1 by quinones [37]. Another line of evidence shows that KEAP1 interacts with NRF2 through a "hinge and latch" mechanism in which the DLG and ETGE motifs of NRF2 bind with the KEAP1 homodimer; since the ETGE motif has a higher affinity than the DLG motif for KEAP1, ETGE acts as a hinge and DLG acts as a latch. NRF2 first binds with KEAP1 through the hinge, then by the latch. Oxidative or electrophilic insults disrupt one or both motifs of NRF2 from KEAP1, which leads to dissociation of NRF2 from KEAP1. As a result, *de novo* synthesized NRF2 accumulates in the cell and this is followed by nuclear translocation and ARE gene expression [38].

From a different perspective, phosphorylation of NRF2 by a series of protein kinases can also lead to changes in the NRF2–KEAP1 complex and subsequent stabilization of NRF2. This was first shown in HepG2 cells using a reporter gene assay where protein kinase C provokes the phosphorylation of NRF2, which directs the dissociation of NRF2 from KEAP1 and promotes the nuclear localization of NRF2 [39]. Similarly, protein kinases such as c-jun N-terminal kinase, extracellular signal-regulated kinase, casein kinase 2, and phosphorylate NRF2, and this leads to the liberation of NRF2 from KEAP1 resulting in overexpression of NRF2 [40–42]. In contrast to the above, several studies have demonstrated that proteins such as p21, p62, WTX tumor suppressor protein, partner and localizer of BRCA2, and DPP3 also promote the dissociation of NRF2 from KEAP1 [43–50].

Interestingly, recent studies have revealed that microRNAs (miRNAs) play a major role in the activation and/or inhibition of NRF2-mediated ARE signaling. miRNAs are a class of small non-coding RNA molecules, 18–25 nucleotides in length, that regulate the post-transcriptional activity of many genes [51]. For the first time, Sangokoya et al. explained the interaction between NRF2 and miRNAs in patients with homozygous sickle-cell disease. They revealed that the miR-144 directly represses NRF2 activity in the K562 cell-line and primary erythroid progenitor cells [52]. Similarly, miR-28 targets the 3′ untranslated region (3′-UTR) of NRF2 mRNA and decreases NRF2-targeted gene expression in a KEAP1-independent manner in breast epithelial cells [53]. However, Eadeset al. showed that miR-200a activates NRF2 in a KEAP1-dependent manner in breast cancer cells; miR-200a targets the 3′UTR of KEAP1 and that leads to the degradation of KEAP1 mRNA [54]. Moreover, Chorley et al. provided ChIP sequencing data which revealed that a wide variety of miRNAs could interact with NRF2 and regulate its transcription. However, more individual experimental data are required to confirm those miRNAs that interact with NRF2 [55]. More recently, Singh et al. showed that NRF2 in turn can also regulate the expression of miRNAs. They found that pentose phosphate pathway (PPP) activity is modulated by miR-1 and miR-206, and NRF2 upregulates the expression of PPP enzymes by increasing the expression of these two miRNA species [56]. However, little is known about NRF2 regulation of these miRNAs.

3. Dual role of NRF2 in carcinogenesis

Several studies on the NRF2–ARE pathway revealed that NRF2 plays a crucial role in cancer chemoprevention and tumor suppression in normal cells. From a different perspective, NRF2 is also considered to play a

major role in tumorigenesis and tumor protection. These two paradoxical roles have been referred as the 'dual role of NRF2'.

3.1. Chemopreventive and tumor-suppressive role of NRF2

Evidence of the chemopreventive role of NRF2 is well-documented. Initially, it was shown that overexpression of NRF2 positively regulates the ARE of the human NQO1 gene in human hepatoblastoma and monkey kidney cells [57]. Further studies using NRF2-deficient mice have shown the critical role of NRF2 in chemoprevention; the degree of GST and NQO1 gene-induction in NRF2-deficient homozygous mice is significantly lower than in NRF2-heterozygous mice [16]. Similarly, NRF2-deficient mice show a significantly increased burden of gastric neoplasia after treatment with a carcinogen such as benzo[a]pyrene than do wild-type mice. In addition, NRF2-knockout mice have lower hepatic and gastric activity of GST and NQO1 than wild-type mice [58]. Furthermore, the incidence of BBN (N-nitrosobutyl(4-hydroxybutyl)amine)-induced urinary bladder carcinoma is significantly higher in NRF2 knockout than in wild-type mice. In addition, the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis and BBN detoxification is mediated by the NRF2–ARE pathway [59]. Similarly, NRF2-knockout mice are susceptible to 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis. The chemopreventive effects of sulforaphane against the carcinogens 7,12-dimethylbenz(a)anthracite or 12-O-tetradecanoylphorbol-13-acetate in skin tumors are mediated through NRF2. Moreover, sulforaphane significantly inhibits skin tumorigenesis in wild-type but not NRF2-null mice [60]. It is noteworthy that, in wild-type mice, the specific hepatic activity of GST and NQO1 is increased by the chemoprotective agent 3H-1, 2-dithiole-3-thione but not in NRF2-knockout mice [61]. Besides, NRF2-null mice show accelerated DNA adduct formation in lung tumors after exposure to diesel exhaust [62]. Further evidence supports the hypothesis that NRF2-deficiency in lung cancer cells creates a microenvironment that facilitates metastasis [63].

Indeed, numerous studies have demonstrated that natural cancer-chemopreventive compounds induce phase-II detoxification enzymes through a mechanism dependent on NRF2–ARE signaling (see the recent review by Magesh et al. [64] for further information on NRF2 activators). In addition, based on a wide variety of studies, several natural and synthetic NRF2 inducers such as isothiocyanates (sulforaphane), curcumin, and dithiolethiones (oltipraz) have been tested in clinical trials for chemoprevention in different cancers [65–67].

3.2. Oncogenic and tumor-protective role of NRF2

Surprisingly, a number of studies have demonstrated that NRF2 protects tumor cells and promotes oncogenesis. These roles have been referred to as the 'dark side of NRF2' [68]. Wang et al. showed that stable overexpression of NRF2 results in enhanced resistance of cancer cells to chemotherapeutic agents such as cisplatin, doxorubicin, and etoposide. Transient transfection of NRF2-small interfering RNA renders cancer cells more susceptible to these drugs. Upregulation of NRF2 by tert-butylhydroquinone also enhances the resistance of cancer cells [68]. Singh et al. showed that NRF2 enhances the expression of drug-efflux pumps (such as ATP-binding cassette, subfamily G, member 2), which in turn facilitates the chemoresistance and tumorigenicity of lung cancer cells [69]. Further experiments showed that oncogenes such as K-Ras, B-Raf, and Myc increase the transactivation of NRF2 that reduces the endogenous ROS level, which leads to the promotion of tumorigenesis [70,71]. Recently, Mitsuishi et al. showed that NRF2 redirects glucose and glutamine into anabolic pathways, and this enables NRF2 to enhance metabolic activity and growth, facilitating tumor-cell proliferation [72]. Interestingly, NRF2 upregulates the transcription of anti-apoptotic proteins such as Bcl-2 and Bcl-xL, leading to decreased apoptosis, enhanced survival, and drug resistance in cancer cells [73,74].

In contrast, RNA-interference-mediated silencing of NRF2 expression in non-small-cell lung cancer A549 cells and prostate cancer cells shows that NRF2 increases the sensitivity to chemotherapeutic drugs and confers radiosensitivity [75–77]. Moreover, constitutive activation of NRF2 protects cancer cells against ionizing radiation and provokes radioresistance in non-small-cell lung cancer cells [78].

Somatic mutations in NRF2 also play a major role in tumorigenesis. When mutations specifically alter amino-acids in the DLG and ETGE motifs of the Neh2 domain, the NRF2-KEAP complex is impaired, and this leads to inhibition of the KEAP1-mediated degradation of NRF2 and constitutive expression of NRF2. This high transcriptional activity of NRF2 occurs in lung, head/neck, esophageal, and skin cancers. In addition, recurrent NRF2 mutation in advanced esophageal squamous cancer confers malignant potential and resistance to chemo/radiotherapy [79–81]. Moreover, Shibata et al. reported that mutant NRF2 induces proliferation, anchorage-independent growth, and tumorigenicity in epithelial cells *via* the mammalian target of rapamycin pathway [82]. Furthermore, Hu et al. identified NRF2 mutations in cells from a patient with non-small-cell lung cancer in which the mutations were located within or near the DLG and ETGE motifs of the Neh2 domain. In addition, they found that all of these mutations were in the purine bases of NRF2 [83].

Several investigations have shown that the loss of KEAP1 function due to somatic mutations leads to constitutive and elevated activation of NRF2–ARE-driven gene expression in cancer cells. Initially, a somatic mutation was identified in the Kelch/DGR domain of KEAP1, where a point-mutation of glycine to cysteine was found. This point-mutation causes a conformational change that reduces its affinity for NRF2, so this loss-of-function of KEAP1 leads to the aberrant activation of NRF2 in cancer cells [84]. Furthermore, somatic mutations of KEAP1 in non-small-cell lung cancer have been identified in highly-conserved amino-acid residues in the Kelch or IVR domain. These mutations lead to decreased KEAP1 function that causes increased accumulation, nuclear translocation, and NRF2–ARE gene expression in lung cancer cells [85]. Similarly, mutations in KEAP1 that lead to the constitutive activation of NRF2 have also been reported in breast, gallbladder, and endometrial cancers, as well as the papillary adenocarcinoma form of lung cancer [86–91].

Besides mutations of KEAP1, its epigenetic regulation is also considered to play a major role in the aberrant activation of NRF2. For the first time, Wang et al. demonstrated that epigenetic changes, such as hypermethylation of CpG sites in the KEAP1 promoter region, lead to down-regulation of KEAP1 expression in human lung cancer cell lines and tissues [92]. In addition, Muscarellaet al. showed that 47% hypermethylation of the promoter region of the KEAP1 gene is present in the tissues of non-small-cell lung cancer patients. Moreover, they predicted that hypermethylation of KEAP1 is more frequent than KEAP1 gene mutations in this cancer [93]. Epigenetic inactivation of the promoter region of the KEAP1 gene has not only been identified in lung cancer but also in prostate [77], malignant glioma [94], colorectal [95], and breast cancer [96]. Collectively, these studies suggest that epigenetic changes in KEAP1 lead to constitutive activation of the NRF2 pathway, which favors the survival of tumors and drug resistance in cancer cells.

A new line of evidence suggests that single-nucleotide polymorphisms (SNPs) in NRF2 also play a critical role in lung cancer. Recently, Suzuki et al. reported that an SNP in the human NRF2 upstream promoter region (rs6721961) of minor A/A homozygotes affects the reduction of NRF2 gene expression in lung cancer. As a result, the risk of lung cancer may be increased even in non-smokers [97]. Likewise, Okano et al. identified SNP-homozygous (c.-617A/A) alleles in the NRF2 gene that are associated with lung adenocarcinoma in non-smoking Japanese women [98].

4. Inhibition of NRF2 by nuclear receptors

It is apparent that an additional regulatory mechanism independent of KEAP1 exists for NRF2. The NRF2 signaling pathway can be regulated by nuclear receptors, which are involved in various physiological

mechanisms in the body (Fig. 3), and this modulation operates in both a ligand-dependent and/or -independent manner (Table 1).

Nuclear receptors are highly-conserved, and are involved in the transduction of extracellular and intracellular signals [99]. Nuclear receptors selectively bind to small lipophilic/hydrophobic ligands that provoke the transactivation of specific cis-regulatory DNA sequences (gene-responsive elements) [100,101]. The binding of ligands to nuclear receptors alters their conformation. As a result, nuclear receptors bind with cofactors such as co-activators or co-repressors that are subsequently responsible for the upregulation or downregulation of their downstream target genes. In contrast, the endogenous ligands of a subset of nuclear receptors have yet to be identified. Those nuclear receptors lacking defined ligands are termed 'orphan' receptors [102]. In humans, so far, 48 nuclear receptors have been identified, while mice have 49 and rats 47 [103]. The nuclear receptor superfamily contains seven subfamilies, NR1–NR6 and NR0 [104].

Most nuclear receptors are similar in their structural organization. In general, they contain six conserved regions designated A to F (Fig. 4). The N-terminal region contains two domains (A/B) termed activation function 1 (AF1); these are the least-conserved domains, are highly variable in length, and exhibit ligand-independent transactivation activity. So far, no three-dimensional structure has been predicted for the A/B domain [105]. Central region C has a DNA-binding domain (DBD) which is said to be the most-conserved region. A pair of zinc fingers in domain C is critically responsible for DNA-specific contacts and interaction. The first zinc finger contains a short motif known as P-box which is responsible for direct DNA interaction and the specificity of DNA-binding, and the second zinc finger contains a D-box which mediates receptor dimerization. Domain D is less conserved among the nuclear receptors; it acts as a flexible hinge between two domains such as C (DBD) and E (a ligand-binding domain). In addition, it contains the nuclear localization signal that regulates the subcellular distribution of nuclear receptors. The E region contains a ligand-binding domain designated activation function 2 (AF2). AF2 has many functions including interaction with lipophilic ligands, activation or repression of the transcriptional activity of nuclear receptors, and dimerization with other nuclear receptors. Finally, the C-terminal region (also known as region-F or the F domain) is highly variable in sequence and little is known about its function [106].

4.1. Retinoids inhibit the NRF2 signaling pathway through retinoic acid receptor alpha

The retinoids are natural and synthetic signaling molecules structurally related to vitamin A. Compounds such as retinol, retinal, retinoic acid (RA), and retinyl esters belong to this group. Retinoids are potent chemopreventive and tumor-suppressive agents because of their apoptotic and anti-oxidant activity. A wide variety of studies in animal models and clinical trials have shown the anticancer activity of RA against lymphoma, leukemia, melanoma, lung cancer, cervical cancer, kidney cancer, neuroblastoma, and glioblastoma [107].

Retinoids induce their physiological effects *via* interaction with two distinct classes of nuclear receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs). These receptors are members of the steroid/thyroid hormone receptor superfamily [108]. The RARs contain three isotypes, RARα, RARβ, and RARγ, encoded by the *RARA*, *RARB*, and *RARG* genes, and function as ligand-dependent transcription factors. There are two important isoforms of RARα (α1 and α2) and RARγ (γ1 and γ2) and five major isoforms of RARβ (β1, β2, β3, β4, and β1′). These isoforms result from the differential use of promoters and alternative splicing [109].

RARs form heterodimers with their most common partners, RXRs. Since RARs are ligand-dependent transcription factors, in the absence of ligand an RAR/RXR heterodimer can interact with multiple co-repressor proteins including the nuclear receptor co-repressor and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) that regulates the transcription of target genes. The RAR/RXR heterodimer co-repressor proteins interact with complexes which have histone deacetylase activity. Subsequently, the histone deacetylases provoke the acetylation of histone tails and target gene expression [110].

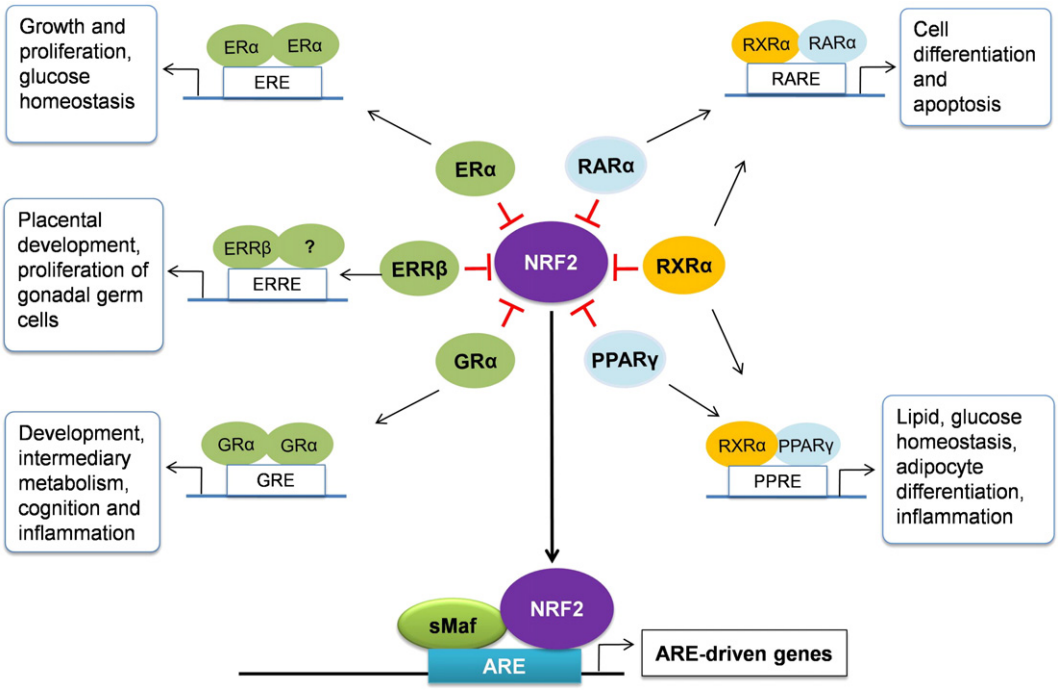

Fig. 3. Repression of NRF2 by nuclear receptors. Nuclear receptors such as RARα, RXRα, PPARγ, ERα, ERRβ, and GR inhibit Nrf2. RARα and RXRα, or PPARγ and RXRα, form heterodimers that bind to either the RA response element or the PPAR-responsive element in various gene promoters. RARα/RXRα is involved in cell differentiation and apoptosis. The PPARγ/RXRα supports adipogenesis. ERα, ERRβ, and GR bind with their target DNA response elements in the promoters of target genes known as ERE, ERRE, and GRE respectively, which are mainly involved in cell growth, proliferation, and metabolism. The exact binding partners and functions of ERRβ are unknown.

Table 1
List of nuclear receptors that inhibit the Nrf2–ARE pathway.

Nuclear receptor	Ligand	Mode of action	Cell/tissue type	Reference
PPARγ	PGJ2 and TRO	Directly binds with NRF2 and abrogates its transactivation	Rat macrophages	[121]
ERα	17β-estradiol	Binds with NRF2 and inhibits its transactivation	MCF-7 breast cancer cells, COS1 cells, Ishikawa cells	[124–127]
RARα	ATRA, AM580	Forms a protein complex with NRF2 and antagonizes its transactivation	MCF-7 breast cancer cells, mouse small intestine	[114]
RARγ	ATRA	Unknown	MCF-7 breast cancer cells	[114]
RXRα	Not tested	DBD of RXRα directly binds with Neh7 domain of Nrf2 and block its transactivation	Human lung carcinoma-A549 cells, Caco2 cells, MCF-7 breast cancer cells, mouse liver	[27]
ERRβ	Unknown	Physically interacts with NRF2 and represses its transactivation	COS-1 cells, Ishikawa cells	[135]
GR	11β-HSD1, Dexamethasone	SMRT binds to Neh4/Neh5 domains of NRF2 and mediates the repression of NRF2 gene expression	H4IIE cells, HEK 293 cells, ARECS3 cells, HPCT-1E3 cells, NIH 3T3 cells, rat liver tissue	[138,139]

Endogenous ligands such as RA act as agonists and activate the RAR/RXR heterodimer complex, leading to a reduction of the affinity between the co-repressor and the complex. Subsequently, coactivator proteins such as steroid receptor coactivators (SRC-1, SRC-2, and SRC-3) and proteins that have histone acetyl transferase activity (such as p300-CBP, P300/CBP-associated factor, and general control of amino-acid synthesis protein 5-like 2) interact with high affinity for the RAR/RXR heterodimer which transactivates RA-targeted genes by binding to downstream DNA response elements known as RA response elements [111]. For instance, the RAR/RXR heterodimer regulates cell growth, differentiation, survival, and death while these nuclear receptors are also implicated in cancer and metabolic diseases such as diabetes and obesity [112,113].

Wang et al. [114] have shown that the induction of ARE-driven luciferase activity is inhibited by all-trans retinoic acid (ATRA) in a stable ARE-luciferase reporter cell line known as AREc32. In addition, ATRA reduces the luciferase activity of ARE genes such as aldo–keto reductase family 1 member C1 (AKR1C1) and AKR1C2 at both the mRNA and protein levels. Interestingly, ATRA does not affect the half-life of NRF2 or its nuclear translocation and accumulation. Treatment of AREc32 cells with the potent NRF2-inducer tert-butylhydroquinone alone or along with ATRA does not have any detectable effect on the blockade of NRF2 nuclear translocation and accumulation. Moreover, these workers found that RARα specifically inhibits the luciferase activity of ARE-driven gene expression. A subsequent study using RNAi knockdown strongly supported the potent antagonistic role of RARα among the isoforms. This study also revealed another isoform, RARγ, that has antagonistic activity on NRF2. However RARγ is not as potent as RARα.

Most importantly, studies using both $NRF2^{+/+}$ and $NRF2^{-/-}$ mice have shown that the expression of the ARE gene battery increases in the small intestine of mice fed a vitamin A-deficient diet, and this increase is repressed by administration of ATRA. By contrast, in the small intestine of NRF2-null mice, the expression of ARE-driven genesis not affected by vitamin A. Altogether, the above findings suggest that ATRA is an endogenous NRF2 inhibitor.

4.2. Mechanism by which retinoid X receptor alpha inhibits NRF2

Similar to RARs, RXRs have three isotypes, RXRα, RXRβ, and RXRγ encoded by the genes *RXRA*, *RXRB*, and *RXRG*. Each isotype has two isoforms, RXRα1 and α2, RXRβ1 and β2, and RXRγ1 and γ2 [115]. The human chromosomal localization of the RXR subtypes is as follows: RXRα on chromosome 9 band q34.3, RXRβ on chromosome 6 band 21.3; and RXRγ on chromosome 1 band q22–q23 [116]. As discussed earlier in this review, RXRs have a structure similar to other nuclear receptors and mediate retinoid signaling by the formation of heterodimers with RARs. In addition, RXRs can form heterodimers with many other nuclear receptors such as those for thyroid hormone, vitamin D, androgen, farnesoid X, and pregnane X, as well as peroxisome proliferator-activated receptors (PPARs) and constitutive androstane receptors [117]. Since RXRs form homo- and hetero-dimers with nuclear receptors and are involved in the RA-induced signaling pathways, based on their potency in different signaling pathways, synthetic RXR-specific ligands (rexinoids) such as Bexarotene have been approved by the Food and Drug Administration (USA) to treat cutaneous T-cell lymphoma and non-small cell lung cancer [118].

Although RXRs heterodimerize with other nuclear receptors, our study specifically established that the heterodimerization of RXRα is not required for NRF2 inhibition [27]. The primary result was that RXRα inhibits both basal and inducible ARE-driven gene expression in MCF7 cells and loss of RXRα increases the transactivation of NRF2-dependent genes. Moreover, forced overexpression of RXRα markedly reduces the mRNA levels of NRF2 target genes such as AKR1C1 and heme oxygenase-1 at both the basal and inducible levels in Caco2 cells in a ligand-independent fashion. Our further knockdown study and GST pull-down assays together revealed that RXRα alone physically binds with NRF2 *in vitro*. Interestingly, the RXRα-NRF2 interaction is completely blocked at amino-acid residues 209–316 [a previously unknown domain of NRF2-Neh7 (NRF2–ECH homology7)]. Moreover, GST pull-down assays provided evidence that the $RXR\alpha^{140–205}$ region that comprises the DBD of RXRα alone is sufficient for interaction

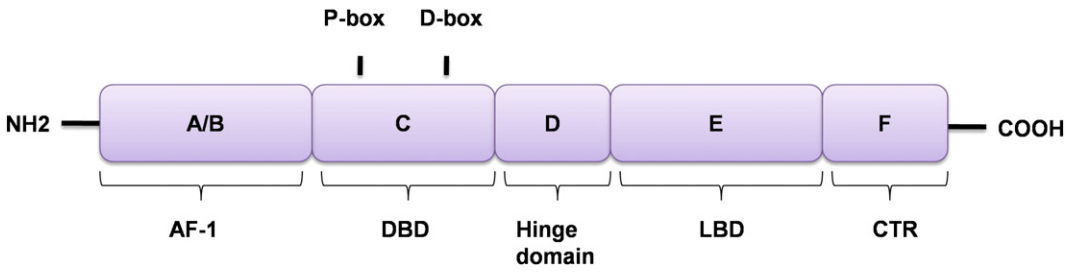

Fig. 4. Common domain structure of nuclear receptors. Nuclear receptors contain six domains, in which A and B constitute the AF-1 region, responsible for ligand-independent transactivation. The C domain (also known as the DBD) is responsible for DNA binding. The pair of zinc fingers known as P-box and D-box is responsible for DNA interaction and dimerization. Domain D acts as a hinge between domains C and E. The E domain is responsible for ligand binding, and the functions of the C-terminal domain (domain F) are unknown.

with the Neh7 domain of NRF2 [27]. A series of additional experiments revealed that RXRα physically binds with NRF2 and forms a heterodimeric protein–protein complex in the nucleus rather than the cytosol. RXRα increases the sensitivity to anticancer drugs such as doxorubicin in A549-RXRα cells. Finally, we hypothesized that binding of RXRα to the Neh7 domain prevents the interaction of CBP with the transactivation domains of NRF2 such as Neh4 and Neh5 because the Neh7 domain lies adjacent to the Neh5 domain and prevents the productive interaction between the transactivation domains of NRF2 and the basal transcription machinery (Fig. 5).

4.3. Antagonism of NRF2 by other nuclear receptors

4.3.1. PPARγ suppresses NRF2 activity

PPARs are nuclear receptors that belong to the thyroid hormone receptor-like subfamily and serve as ligand-dependent transcription factors [119]. They have three isoforms: PPARα, PPARβ, and PPARγ. They heterodimerize with RXRs and regulate the transcription of various genes by interacting with the PPAR-responsive element. The major functions of PPARs include lipid and glucose homeostasis, differentiation of adipocytes, and regulation of inflammation [120]. Thromboxane synthase (TXS) is a member of the cytochrome P450 superfamily of enzymes and plays a crucial role in cellular hemostasis. For the first time, Ikeda et al. reported transcriptional regulation of the rat TXS gene by PPARγ in macrophages. During this study, they found downregulation of TXS gene expression by PPARγ ligands such as 15-deoxy-D12, 14-prostaglandin J2 and thiazolidinedione troglitazone. In addition, overexpression of PPARγ suppresses TXS gene transcription. Furthermore, they identified the element responsible for the PPARγ effect on the TXS gene, the NF-E2/AP-1 site, as well as reporting that PPARγ directly interacts with NRF2 and represses its transactivation [121].

4.3.2. Repression of NRF2 by estradiol (E2)-bound estrogen receptor alpha (ERα)

The estrogen receptor is a ligand-dependent transcription factor induced by the hormone estrogen. There are two estrogen receptor types, ERα and ERβ. It is well known that the DBDs of these receptors are ~97% homologous [122]. These receptors bind with their respective estrogen response elements and play a crucial role in different cancers including prostate, breast, uterus, ovary, colon, lung, and stomach cancers, so they are considered as drug targets in cancer therapy. ERα is specifically involved in functions such as growth, proliferation, and glucose homeostasis [123].

Ansell et al. first demonstrated that E2 and other estrogens downregulate the activity of phase-II enzymes both *in vitro* and *in vivo* [124]. In addition, they proposed that this downregulation increases the oxidative DNA damage that results in the progression of cancer in some estrogen-responsive tissues. Furthermore, another study by the same group revealed that estrogens repress ARE-driven gene transcription and enhance cancer progression in an NRF2-dependent manner, and this is independent of Keap1-mediated degradation. Moreover, immunoprecipitation revealed a physical interaction between ERα and NRF2 which is specifically dependent on ligands such as E2. In addition, ERα represses NRF2-mediated transcription through either the A/B (AF1) or the C domain [125].

Other studies have shown that inhibition of estrogen signaling leads to activation of the NRF2 pathway in breast cancer. Yao et al. reported that the ERα ligand-binding domain is required for the estrogen-dependent inhibition of NQO1 promoter activity in breast cancer cells. The results of ChIP assays have shown that estrogen recruits ERα and a class-III histone deacetylase (SIRT1) at the NQO1 promoter, leading to the inhibition of NQO1 transcription. In addition, anti-estrogen shikonin-induced inhibition of ERα expression reverses the inhibitory action of estrogen on NQO1 expression [126]. More recently, Lo et al. also reported that E2-induced ERα represses activity of the NQO1 and HMOX1 genes in an NRF2-dependent manner in MCF-7 breast cancer cells [127].

4.3.3. ERRβ is a potent antagonist of NRF2

Estrogen-related receptors (ERRs) are members of the nuclear hormone receptor family of steroid hormone receptors and contain three isotypes, ERRα, ERRβ, and ERRγ, that were initially discovered as orphan receptors [128]. ERRs are thought to be closely related to estrogen receptors and share similar target genes, co-regulatory proteins, and sites of action [129]. Since ERRβ is an orphan receptor, little is known about its function. However, a growing body of evidence suggests that it plays a major role in early placental development [130], the proliferation of gonadal germ cells [131], the development and/or function of endolymph-producing epithelia [132], tumor-suppression in prostate cancer cells [133], and rod photoreceptor survival [134].

Zhou et al. showed that human ortholog of ERRβ-short-form hERRβ (SFhERRβ) inhibits NRF2 transcriptional activity [135]. The results of ARE-luciferase assays revealed that SFhERRβ inhibits the transcriptional activity of HA-NRF2 (hemagglutinin-tagged NRF2), and increasing the level of a SFhERRβ expression plasmid leads to the repression of tertbutylhydroquinone-induced luciferase activity in COS-1 cells. Moreover, this result established that SFhERRβ is a more potent inhibitor of HA-NRF2 transactivation than other isoforms such as hERRα and hERRγ. Furthermore, they have shown that overexpression of SFhERRβ represses the endogenous NRF2-dependent ARE gene expression at both the basal and inducible levels. Co-immunoprecipitation experiments specifically revealed that SFhERRβ physically interacts with NRF2 and represses its activity. Confocal immunofluorescence microscopy established that SFhERRβ alters the subcellular localization of NRF2. Additional analyses using SFhERRβ deletion mutants have shown that SFhERRβ binds with NRF2 at multiple sites. Therefore, the overall experimental data suggest that ERRβ exerts potent inhibitory activity on NRF2-targeted gene expression.

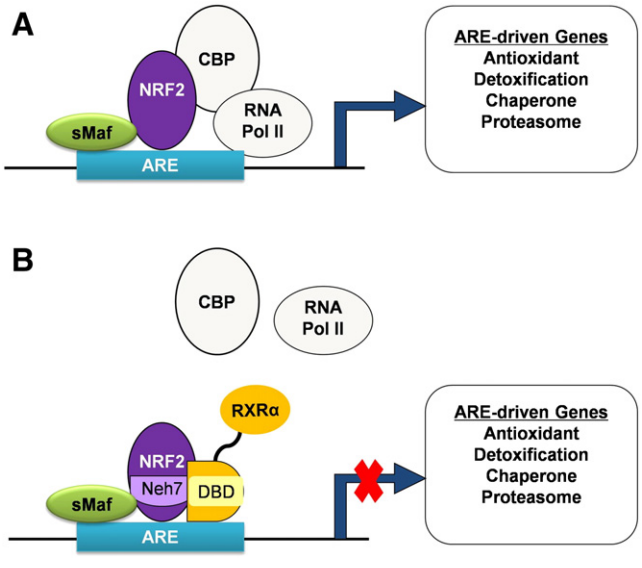

Fig. 5. Inhibition of NRF2 by the DBD of RXRα through the Neh7 domain. (A) In the absence of RXRα, NRF2 along with Maf protein is associated with the transcription machinery that includes CBP and RNA polymerase II that lead to the activation of ARE genes. (B) The DBD of RXRα interacts with the Neh7 domain of NRF2 that prevents binding of the transcription elements CBP and RNA polymerase II and ultimately provokes the inhibition of ARE gene expression.

4.3.4. Glucocorticoid receptor inhibits NRF2 activity

The glucocorticoid receptor (GR; also known as NR3C1) which belongs to the nuclear receptor subfamily 3, is a ligand-induced transcription factor, and is activated by the steroid hormones known as glucocorticoids [136]. GRs regulate a number of genes through direct

binding to their target DNA motifs called glucocorticoid-responsive elements and play an important role in the regulation of a wide range of physiological processes, such as immunity, intermediary metabolism, skeletal growth, cardiovascular function, reproduction, and cognition [137]. For the first time, Ki et al. [138] investigated the repression of NRF2-dependent GSTA2 gene activity by GRs. Dexamethasone (Dex), a potent synthetic glucocorticoid that activates GRs, inhibits GSTA2 expression constitutively induced by oltipraz or t-BHQ in H4IIE cells. In addition, Dex-GR activation represses GSTA2 promoter-luciferase gene activity but does not inhibit the nuclear translocation and oltipraz- or t-BHQ-induced DNA activity of NRF2. Interestingly, deletion of the glucocorticoid response element in the GSTA2 promoter suppresses the inhibitory activity of Dex. A series of experiments in their study revealed that SMRT, a co-repressor of the steroid–GR complex, binds with the Neh4/5 domain of NRF2 to mediate the repression of NRF2-dependent gene expression. An additional small-interfering RNA study of SMRT also clearly showed that it directly binds to the transactivation domains of NRF2 (Neh4/Neh5). They hypothesized that the binding of SMRT, recruited to the steroid–GR complex on the glucocorticoid response element, negatively regulates GSTA2 gene transcription with NRF2 activation. These findings revealed that ligand-dependent GR activation may lead to the repression of NRF2–ARE gene expression.

Recently, Kratschmar et al. showed that 11β-HSD1 (11β-hydroxysteroid dehydrogenase type 1) activates GRs through glucocorticoids and leads to suppression of the NRF2-dependent antioxidant response. Using transfected HEK-293 cells and hepatic H4IIE cells they found that, when cells expressing 11β-HSD1 are treated with cortisone, the marker genes NQO1, HMOX1, and GST2A are downregulated. At the same time, a reversal effect on the suppression of NRF2-dependent genes was found on treatment with 11β-HSD1 inhibitors. Furthermore, they demonstrated that elevated glucocorticoids reduce the ability of cells to detoxify H_2O_2 [139]. These observations clearly demonstrated that 11β-HSD1-induced GR activation impairs the NRF2-dependent antioxidant response.

5. Biological significance of NRF2 regulation by nuclear receptors

Nuclear receptors are believed to control essential physiological functions and are predominantly present in the nucleus, where they bind with their respective DNA sequences (*e.g.* hormone-responsive elements) and regulate gene expression [140]. Nuclear receptors have been implicated in cancer, as well as metabolic and other diseases. Hence, nuclear receptors are emerging as drug targets to treat these diseases [105]. Likewise, NRF2 also plays an important role in cancer and metabolic diseases. The regulation of nuclear receptors is mediated in a ligand-dependent and a ligand-independent fashion. For instance, ERα and RXRα physically interact with NRF2, form a protein–protein complex, and negatively regulate ARE gene expression where ERα, but not RXRα, requires the ligand estrogen. Conversely, downregulation of nuclear receptors such as RXRα has been reported in several tumors, especially non-small cell lung cancer [141–143]. It is noteworthy that most of the studies of aberrant NRF2 activation have been carried out in non-small cell lung cancer cell lines. Similar to NRF2, some nuclear receptors play dual roles in the etiology of cancer. For example, loss of estrogen or its receptors contributes to the development or progression of various tumors. On the other hand, overexpression of estrogen receptors can lead to the development of different cancers, specifically breast cancer. Hence, both activation and inhibition of estrogen receptors is required to modulate gene expression in different cancers [144]. Another nuclear receptor, PPARγ, plays a dual role in cancers. It has been reported that PPARγ acts as both a tumor inhibitor and a tumor promoter in cancers of the colon, breast, urinary tract, and lung [145]. These findings show that a distinct regulatory mechanism seems to exist between NRF2 and nuclear receptors, and this is of considerable interest.

Indeed, it is interesting to note that several other receptors including ERα [125,126], ERRβ [135], and PPARγ [121] repress NRF2. Repression of NRF2 by nuclear receptors serves to limit the antioxidant capacity of cells and influence their redox status. It may also influence the metabolism and disposition of xenobiotics. For example, loss of NRF2 activity could diminish the oxidation and reduction reactions catalyzed by enzymes such as aldo–keto reductases and NQO1 in favor of those catalyzed by other phase-I detoxification enzymes, or it may decrease the elimination of xenobiotics by transporters associated with multi-drug resistance. It is possible that some of the enzymes or drug transporters encoded by NRF2-target genes decrease the half-life of nuclear receptor ligands or their disposition. It could therefore be envisaged that for nuclear receptor ligands to be efficient, it might be advantageous to attenuate NRF2 activity.

Despite nuclear receptor-DNA interactions, an additional mechanism that involves protein–protein interactions of nuclear receptor–NRF2 could play an important role in hormone-related diseases. Most of the ligands of nuclear receptors are hormones that play crucial roles in numerous bodily functions. Recent evidence showed that NRF2 not only has adverse effects in cancer but also in atherosclerosis [146]. In addition, there may be as-yet unknown deleterious effects associated with aberrant NRF2 activation in other diseases. Hence, the interplay between these two major signaling pathways allows fine-tuning of the regulation of essential biological processes. Thus, a better understanding of the regulation of NRF2 by nuclear receptors may allow opportunities for the development of new treatments to inhibit constitutive activation of the NRF2-ARE pathway.

6. Therapeutic perspective for inhibiting NRF2 through nuclear receptors

So far, most research has focused on the activation of NRF2, but less was known about its inhibition until the discovery of its oncogenic role. However, the molecular mechanism involved in the role of NRF2 in carcinogenesis still needs to be investigated. Meanwhile, in order to overcome the NRF2-based drug resistance in cancer therapy, inhibition of NRF2 is likely to become a new therapeutic approach. Recently, several laboratories including ours have identified several NRF2 inhibitors including endogenous proteins such as Bach1 [147], p53 [148], ATRA [114], activating transcription factor 3 [149], E-cadherin [150], and caveolin-1 [151] as well as nuclear receptors. In addition, exogenous inhibitors such as ochratoxin-A [152], luteolin [153], brusatol [154], procyanidin [155], apigenin [156], chrysin [157], and trigonelline [158] potently inhibit the NRF2 signaling pathway.

Coincidentally, a recent study has confirmed the importance of the Neh7 domain of NRF2 where caveolin-1 binds to the 281–289amino-acid region and inhibits its transactivation [151]. Most nuclear receptors are considered to be potential pharmacological targets for drug discovery. Specific ligands or modulators have been shown to target nuclear receptors for transcriptional regulation [159]. It is noteworthy that regulation of NRF2 is required in the nucleus rather than the cytosol because the transactivation of ARE genes is processed in the nucleus. Thus, given the contribution of nuclear receptors to the inhibition of NRF2, we conclude that targeting the Neh7 domain of NRF2 for its inhibition by nuclear receptors may become a novel therapeutic approach in cancer treatment.

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