BIOMOLECULES & THERAPEUTICS

Invited Review
Biomol Ther 26(1), 57-68 (2018)

Dysregulation of NRF2 in Cancer: from Molecular Mechanisms to Therapeutic Opportunities

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Abstract

Nuclear factor E2-related factor 2 (NRF2) plays an important role in redox metabolism and antioxidant defense. Under normal conditions, NRF2 proteins are maintained at very low levels because of their ubiquitination and proteasomal degradation via binding to the kelch-like ECH associated protein 1 (KEAP1)-E3 ubiquitin ligase complex. However, oxidative and/or electrophilic stresses disrupt the KEAP1-NRF2 interaction, which leads to the accumulation and transactivation of NRF2. During recent decades, a growing body of evidence suggests that NRF2 is frequently activated in many types of cancer by multiple mechanisms, including the genetic mutations in the KEAP1-NRF2 pathway. This suggested that NRF2 inhibition is a promising strategy for cancer therapy. Recently, several NRF2 inhibitors have been reported with anti-tumor efficacy. Here, we review the mechanisms whereby NRF2 is dysregulated in cancer and its contribution to the tumor development and radiochemoresistance. In addition, among the NRF2 inhibitors reported so far, we summarize and discuss repurposed NRF2 inhibitors with their potential mechanisms and provide new insights to develop selective NRF2 inhibitors.

Key Words: NRF2, KEAP1, NRF2 inhibitors, Cancer

INTRODUCTION

Nuclear factor E2-related factor 2 (NRF2), also known as nuclear factor (erythroid-derived 2)-like 2 (NFE2L2), belongs to the Cap'n'Collar/basic leucine zipper (CNC-bZIP) family of transcription factors (Moi *et al*., 1994). NRF2 plays a pivotal role in maintaining redox homeostasis by inducing the expression of a wide array of genes involved in antioxidant defense (Hayes and Dinkova-Kostova, 2014; Tebay *et al*., 2015). The NRF2 protein contains seven highly conserved NRF2-ECH homology (Neh) domains, among which Neh1, 3, 4, and 5 are involved in the activation, whereas Neh2, 6, and 7 are involved in the inhibition (Fig. 1A). Neh1 is a CNC-bZIP domain that binds to a small musculoaponeurotic fibrosarcoma (sMAF) and DNA promoter region (Itoh *et al*., 1997). Neh3, 4, and 5 are transactivation domains that interact with transcriptional coactivators. The Neh3 domain binds to chromo-ATPase/helicase DNA binding protein 6 (CHD6) (Nioi *et al*., 2005), while Neh4 and 5 bind to the CREB binding protein (CBP) (Katoh *et al*., 2001). Moreover, the Neh5 domain contains a redox-sensitive nuclear-export signal (NES) that regulates the intracellular localization of NRF2 (Li *et al*., 2006). Neh2 and Neh6 are required for ubiquitination and proteasomal degradation of NRF2. The Neh2 domain binds to kelch-like ECH associated protein 1 (KEAP1), which is an adaptor protein of NRF2 in the cullin3 (CUL3)- ring-box 1 (RBX1)-based E3 ubiquitin ligase complex (Katoh *et al*., 2005; Tong *et al*., 2006). Neh6 is a serine-rich domain harboring DSGIS and DSAPGS motifs that bind to β-transducin repeat-containing protein (β-TrCP) in the CUL1-s-phase kinase associated protein 1 (SKP1)-RBX1-based E3 ubiquitin ligase complex (Rada *et al*., 2011; Chowdhry *et al*., 2013). Notably, prior serine phosphorylation by glycogen synthase kinase 3 (GSK3) on the DSGIS motif in the Neh6 domain is required for β-TrCP recognition. The Neh7 domain was recently identified as a retinoid X receptor α (RXRα) binding domain, which leads to inhibition of NRF2 (Wang *et al*., 2013).

KEAP1 is a cysteine-rich and redox-sensitive protein containing five functional domains, which include an N-terminal region (NTR), a broad-complex, tramtrack, bric a′ brac (BTB) homodimerization domain, a cysteine rich intervening region (IVR), a kelch/double glycine repeat (DGR) domain (harboring six Kelch repeats), and a C-terminal region (CTR) (Itoh *et al*., 2010) (Fig. 1B). The BTB domain is important for KEAP1

Open Access **https://doi.org/10.4062/biomolther.2017.195**

Received Sep 27, 2017 **Revised** Oct 19, 2017 **Accepted** Oct 24, 2017
Published Online Dec 7, 2017

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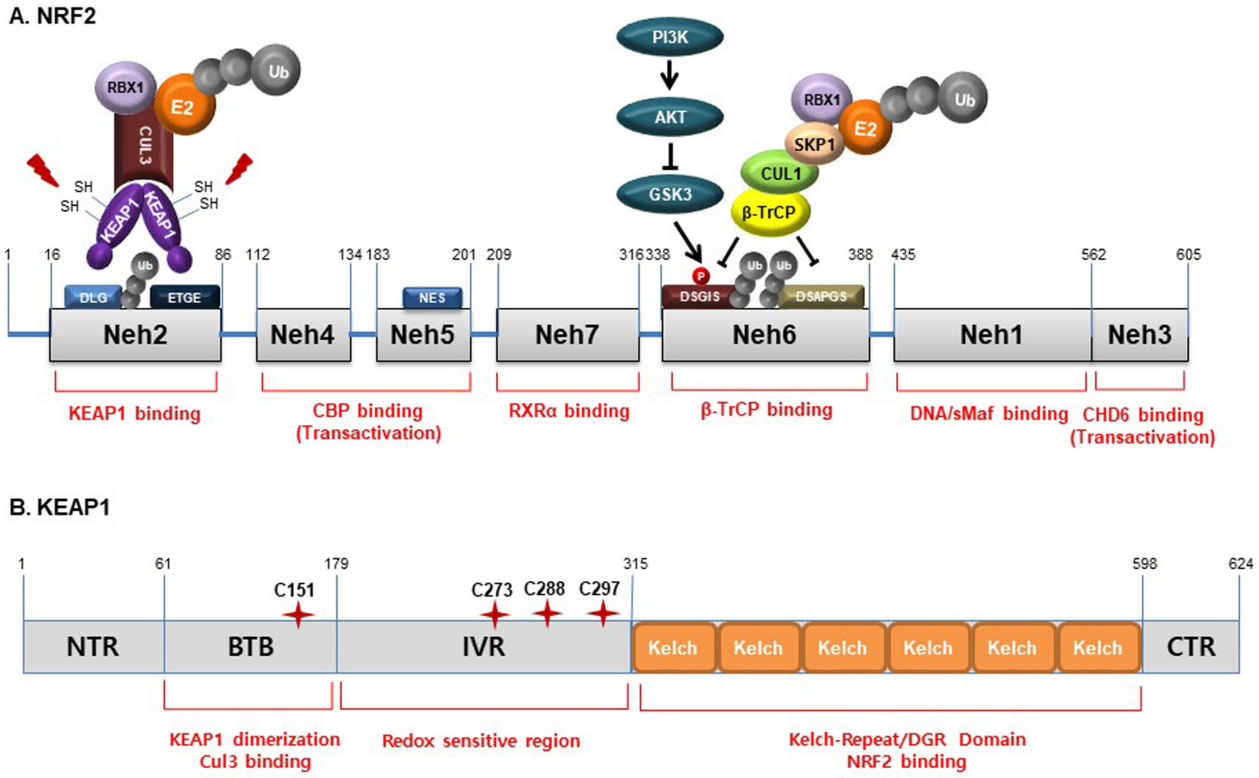

Fig. 1. Structural function and regulation of nuclear factor E2-related factor 2 (NRF2) and kelch-like ECH associated protein 1 (KEAP1) proteins. (A) Domain structure of NRF2. (B) Domain structure of KEAP1. Cysteine residues critical for KEAP1 dimerization (C151) and redox sensing (C273, C288, C297) are indicated.

homodimerization and interaction with the CUL3-based E3 ubiquitin ligase complex (Zipper and Mulcahy, 2002; Furukawa and Xiong, 2005). The IVR domain contains highly reactive cysteine residues, such as Cys273, Cys288, and Cys297, which are easily oxidized and are thus responsible for sensing oxidative stress (Dinkova-Kostova *et al*., 2002). The DGR domain contains six repetitive kelch structures that specifically bind to the Neh2 domain of NRF2 (Itoh *et al*., 1999).

In normal conditions, KEAP1 plays a major role in restraining NRF2 activity by binding to the DLG/ETGE motifs in the Neh2 domain and inducing ubiquitination and proteasomal degradation of NRF2 (Itoh *et al*., 2010) (Fig. 2). Upon oxidative and/or electrophilic stress, highly reactive cysteine residues in KEAP1 are oxidized, which prevents KEAP1 from binding to NRF2 for ubiquitination (Zhang *et al*., 2004). Consequently, NRF2 is accumulated and translocated into the nucleus where it heterodimerizes with sMAF via its Neh1 domain and binds to antioxidant response element (ARE), inducing the transactivation of its target genes (Taguchi *et al*., 2011) (Fig. 2). The majority of NRF2's targets encode metabolic enzymes regulating redox homeostasis by detoxifying reactive oxygen species (ROS) or electrophiles, and repairing the oxidative damage. Thus, promoting anti-oxidant defense in normal cells by activating NRF2 has been considered an attractive and promising strategy to prevent cancer development (Kwak and Kensler, 2010).

However, importantly, recent studies have shown that NRF2 is frequently activated by multiple mechanisms with potent oncogenic effects in cancer (Leinonen *et al*., 2015; Menegon *et al*., 2016; Taguchi and Yamamoto, 2017). Analysis in The Cancer Genome Atlas (TCGA) showed that genetic mutations leading to the activation of NRF2 were found in more than 20% of lung adenocarcinomas (LUAD) and 34% of lung squamous cell carcinomas (LUSC) (Cancer Genome Atlas Research Network, 2012, 2014). Accumulating evidence suggests that the activation of NRF2 is critical for tumor cell proliferation, growth, and survival (Ohta *et al*., 2008; DeNicola *et al*., 2011; Mitsuishi *et al*., 2012; Jia *et al*., 2016). Moreover, NRF2 activation is thought to be the main cause of resistance to chemotherapy and radiotherapy (Ramos-Gomez *et al*., 2001; Singh *et al*., 2006; Shibata *et al*., 2008a; Jiang *et al*., 2010; Zhang *et al*., 2010; Zhou *et al*., 2013; No *et al*., 2014; Choi and Kwak, 2016; Ryoo *et al*., 2016). Thus, these data strongly suggest that inhibition of NRF2, either alone or in combination, could be a promising therapeutic strategy for cancer. However, currently, NRF2 inhibitors are neither clinically available nor under clinical trial. Recently, several NRF2 inhibitors have been reported to have promising therapeutic efficacy (Zhu *et al*., 2016). In this review, we summarize the currently-known mechanisms of NRF2 dysregulation in cancer. We also summarize the NRF2 inhibitors particularly focused on the repurposed one reported so far and discuss their potential mechanisms and future directions to develop selective NRF2 inhibitors.

MECHANISMS OF NRF2 ACTIVATION IN CANCER

In normal cells, the KEAP1-CUL3-RBX1 complex plays a

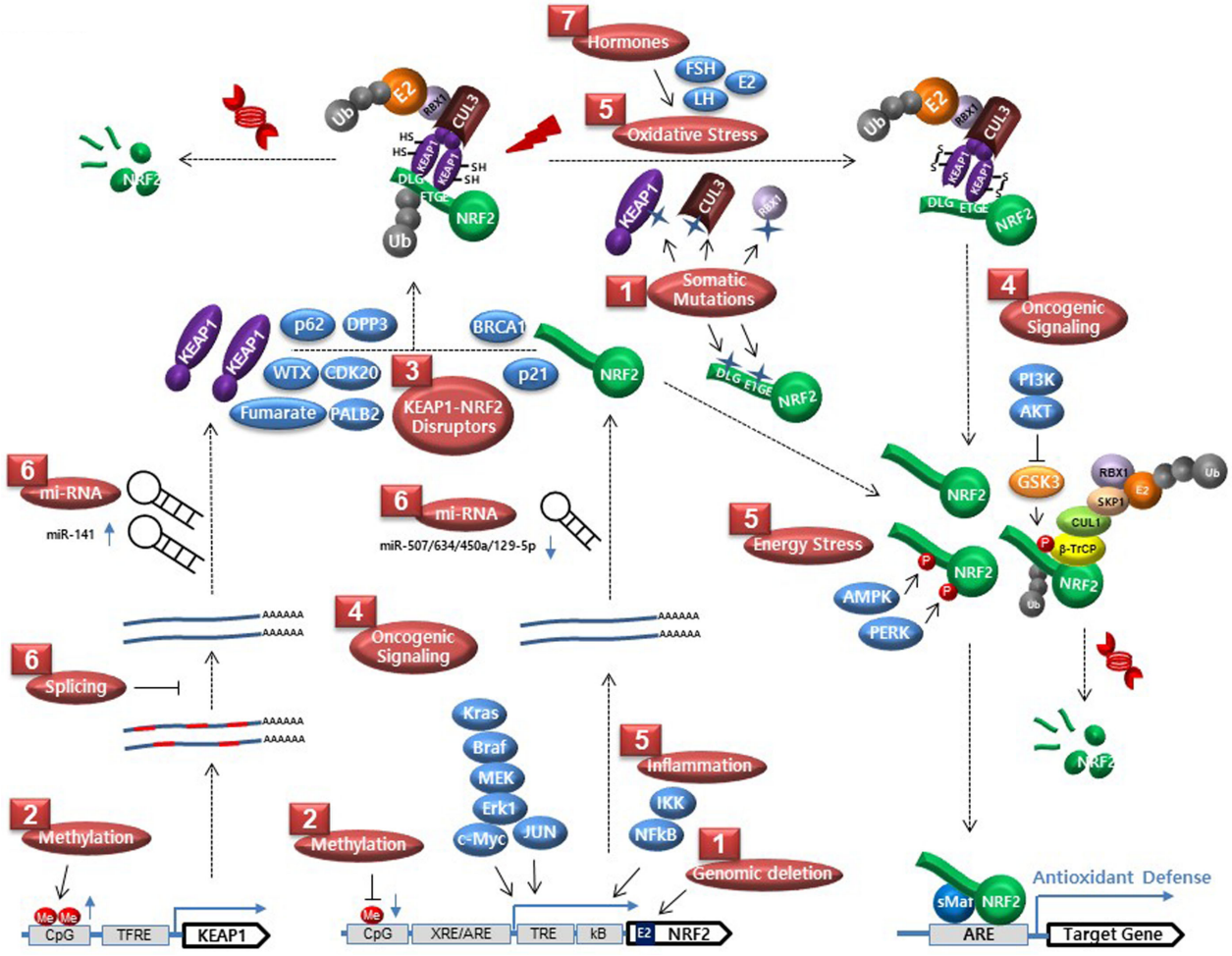

Fig. 2. Seven mechanisms of nuclear factor E2-related factor 2 (NRF2) activation in cancer. (1) Genetic mutations, (2) Epigenetic modifications, (3) KEAP1-NRF2 disruption, (4) Oncogenic signaling, (5) Stress signaling, (6) RNA processing, (7) Hormonal activation.

central role in regulating NRF2 activity by inducing ubiquitination and proteasomal degradation of NRF2, keeping the protein levels very low (Fig. 2). However, in cancer, this tight regulation of the KEAP1-NRF2 pathway has been reported to be compromised by multiple mechanisms discussed below (Fig. 2).

Genetic mutations

Somatic mutations of the genes involved in the KEAP1-NRF2 pathway comprise the most well-known mechanism of NRF2 activation in cancer (Sporn and Liby, 2012; Menegon *et al*., 2016). Recently, large-scale cancer genome projects, such as TCGA, have provided comprehensive characterization of genomic alterations in the KEAP1-NRF2 pathway. In Lung Adenocarcinoma (LUAD), loss of function mutations in *KEAP1* and *CUL3* leading to the activation of *NRF2* were found in 19% and less than 1%, respectively, while gain of function mutations in *NRF2* were found in 3% of patients with cancer (Cancer Genome Atlas Research Network, 2014). By contrast, in lung squamous cell carcinoma (LUSC), loss of function mutations in *KEAP1* and *CUL3* leading to the activation of NRF2 were found in 12% and 7% respectively, while gain of function mutations in *NRF2* were found in 19% of patients with cancer (Cancer Genome Atlas Research Network, 2012). In addition to lung cancer, mutations in *KEAP1* or *NRF2* have been found in diverse cancer types, such as breast cancer (Sjöblom *et al*., 2006; Nioi and Nguyen, 2007), gastric cancer, colorectal cancer, prostate cancer (Yoo *et al*., 2012), gall bladder cancer (Shibata *et al*., 2008a), ovarian cancer (Konstantinopoulos *et al*., 2011), liver cancer (Guichard *et al*., 2012; Cleary *et al*., 2013; Fujimoto *et al*., 2016), and esophageal carcinoma (Kim *et al*., 2010; Shibata *et al*., 2011). Notably, in contrast to the *KEAP1* mutations, which occur throughout the gene and are either missense or nonsense mutations (Singh *et al*., 2006; Ohta *et al*., 2008), all the mutations in NRF2 are found exclusively within regions encoding the DLG/ETGE motifs, which prevent KEAP1 binding (Shibata *et al*., 2008b). Recently, recurrent loss of *NRF2* exon 2 was reported as a novel mechanism for the activation of NRF2 in lung cancer and head and neck cancer (Goldstein *et al*., 2016). Loss of exon 2 from the *NRF2* gene results in the synthesis of an NRF2 protein missing the KEAP1 interacting domain, thereby inducing NRF2 accumulation and transcriptional activation of its target genes. In addition, the loss of function mutations in *CUL3* and *RBX1* leading to the activation of NRF2 have been reported frequently in sporadic papillary renal cell carcinoma (PRCC)

(Ooi *et al*., 2013) and serous ovarian cancer (Martinez *et al*., 2014), respectively.

Epigenetic modifications

Epigenetic modifications in *KEAP1* and *NRF2* promoter regions contribute to the activation of NRF2 in cancer. The promoter region of *KEAP1* is hypermethylated in several cancers, including lung (Wang *et al*., 2008; Muscarella *et al*., 2011), colon (Hanada *et al*., 2012), and prostate cancers (Zhang *et al*., 2010), leading to the reduction of *KEAP1* expression and the accumulation of NRF2. Importantly, methylation within the *KEAP1* promoter region in patients with glioma is associated with poor prognosis. Recently, demethylation of *NRF2* promoter regions resulting in the overexpression of *NRF2* was also reported in drug-resistant colon cancer cells (Zhao *et al*., 2015). These observations suggest that reversal of *KEAP1* methylation or *NRF2* demethylation would inhibit *NRF2* expression, which might contribute to a better outcome of chemotherapy.

KEAP1-NRF2 disruptors

Accumulation of KEAP1-NRF2 disrupting proteins and metabolites can activate NRF2 in cancer. p62, also known as sequestosome 1 (SQSTM1), is the most well-known disruptor, which competes with NRF2 for directly binding to KEAP1 through an STGE motif that is similar to the ETGE motif in NRF2 (Copple *et al*., 2010; Jain *et al*., 2010; Komatsu *et al*., 2010; Lau *et al*., 2010). Once bound to KEAP1, p62 induces autophagic degradation of KEAP1 (Komatsu *et al*., 2010). Importantly, recent studies have shown that p62 is upregulated in hepatocellular carcinoma (HCC) and p62-induced activation of NRF2 is critical for HCC development (Inami *et al*., 2011; Umemura *et al*., 2016), which supports the physiological significance of the p62-NRF2 axis in cancer development. Similarly, dipeptidyl-peptidase 3 (DPP3) (Hast *et al*., 2013), encoded by a Wilms tumor gene on the X chromosome (WTX) (Camp *et al*., 2012), and partner and localizer of BRCA2 (PALB2) (Ma *et al*., 2012) have been shown to disrupt the KEAP1-NRF2 interaction by competing with NRF2 for binding to KEAP1. Importantly, a recent study showed that DPP3 is overexpressed in breast cancer and its expression correlates with NRF2 downstream gene expression and poor prognosis, particularly in estrogen receptor-positive cancer (Lu *et al*., 2017). Recently, cyclin-dependent kinase 20 (CDK20) was identified as a novel KEAP1-interacting protein, which competes with NRF2 for KEAP1 binding through its N-terminal ETGE motif (Wang *et al*., 2017). Importantly, CDK20 is overexpressed in lung cancer tissues and is critical for promoting cell proliferation and radiochemoresistance in lung cancer. In addition, p21 and breast cancer 1 (BRCA1) were shown to compete with KEAP1 for binding to the ETGE and/or DLG motifs of NRF2 (Chen *et al*., 2009; Gorrini *et al*., 2013).

In addition to proteins, oncometabolite fumarate can also activate NRF2 by interrupting the KEAP1-NRF2 interaction. Deficiency of the tricarboxylic acid cycle enzyme, fumarate hydratase (FH), in type 2 PRCC induces the accumulation of fumarate, which induces succinylation of cysteine residues in KEAP1, resulting in the accumulation of NRF2 (Adam *et al*., 2011; Ooi *et al*., 2011). This activation of NRF2 was shown to be critical for growth and survival of FH-deficient PRCC.

Oncogenic signaling

Oncogenic signaling pathways can drive NRF2 activation in cancer. Kirsten rat sarcoma viral oncogene homolog (K-Ras), one of the most activated oncogenes in cancer was shown to increase *NRF2* transcription via activation of the B-Raf-MEK-ERK (V-Raf-1 murine leukemia viral oncogene homolog B–mitogen-activated protein kinase kinase) signaling pathway (DeNicola *et al*., 2011). Moreover, they showed that the activation of K-Ras and B-Raf stimulates the transcription of NRF2 via activation of transcription factors Jun and Myc. Recently, another group showed that K-Ras-ERK signaling pathway increases NRF2 transcription through TPA (12-O-Tetradecanoylphorbol-13-acetate) response element (TRE) reside in a regulator region in exon 1 of NRF2 (Tao *et al*., 2014). Importantly, this activation of NRF2 was shown to be critical for tumor growth and enhanced chemoresistance of K-Ras mutant cancer cells (DeNicola *et al*., 2011; Tao *et al*., 2014). In addition, the phosphatidylinositol-4,5-bisphosphate 3-Kinase (PI3K)-serine/threonine kinase (AKT) signaling pathway can also induce NRF2 accumulation, either through an increase in *NRF2* transcription (Mitsuishi *et al*., 2012), nuclear accumulation (Madduma Hewage *et al*., 2017), or inhibition of GSK3-β-TrCP-induced proteasomal degradation of NRF2 (Chowdhry *et al*., 2013).

Stress signaling

The tumor microenvironment can be characterized as a stressful condition, where tumor cells encounter inflammation, oxidative stress, and nutrient starvation (Koumenis *et al*., 2014). Oxidative stress is a well-known inducer of NRF2 activation through cysteine oxidation and inhibition of KEAP1. Interestingly, accumulating data suggest that inflammation and nutrient deficiency also activate NRF2 in tumor cells. It was shown that lipopolysaccharide (LPS) induced NRF2 transcription via activation of NF-κB, which directly binds to κB site within the promoter region of NRF2 (Rushworth *et al*., 2008, 2012; Liu *et al*., 2017). Moreover, NRF2 is consitutively active in human acute myeoloid leukemia (AML) cells via activation of NF-κB and conferred chemoresistance in AML suggesting that inflammation can induce chemoresistance via activation of NRF2. In addition, glucose deprivation induced ER stress-dependent activation of PRKR-like endoplasmic reticulum kinase (PERK), which in turn phosphorylates and activates NRF2 (Cullinan *et al*., 2003; Cullinan and Diehl, 2004; Ding *et al*., 2016). In addition, 5'-AMP-activated protein kinase (AMPK), which is activated under energy stress conditions (Jeon, 2016), can phosphorylate and activate NRF2 by inducing its nuclear accumulation (Joo *et al*., 2016). Another group showed that AMPK can also indirectly activate NRF2 by reducing endoplasmic reticulum (ER) stress (Zimmermann *et al*., 2015), which is inconsistent with another study that showed positive effect of ER-stress on NRF2 activation via PERK, as mentioned above. Thus, further studies are required to understand the role of ER-stress on NRF2 regulation. Collectively, considering that oxidative stress, inflammation, and nutrient deficiency in tumor microenvironment are activators of NRF2 as well as AMPK in tumors (Jeon and Hay, 2012, 2015), hyperactivation of NRF2 would be a common phenomenon in most tumors *in vivo*, even in the absence of other alterations in the KEAP1-NRF2 pathway.

RNA processing

NRF2 activation can also occur at the post-transcriptional level in cancer through abnormal regulation of microRNA

(miRNA) or mRNA splicing. Among the downregulated miRNAs in esophageal squamous cell carcinoma (ESCC), four miRNAs, miR-507, miR-634, miR-450a, and miR-129-5p, directly target and inhibit the expression of *NRF2* and are associated with poor prognosis (Yamamoto *et al*., 2014). MiR-141, which targets *KEAP1* and induces NRF2 accumulation, is upregulated in cisplatin-resistant ovarian cancer and 5-fluorouracil (5-FU)-resistant HCC and contributes to chemoresistance (van Jaarsveld *et al*., 2013; Shi *et al*., 2015). In addition, abnormal splicing of *KEAP1* mRNA, resulting in nonfunctional KEAP1 protein that is unable to restrain NRF2, was reported in colon cancer cells (Zhang *et al*., 2010). These observations suggest that NRF2 can be also activated during *KEAP1* or *NRF2* mRNA processing.

Hormonal activation

Lastly, hormonal activation of NRF2 has been reported in ovarian cancer. Compared with benign ovarian tumor, ovarian carcinoma overexpresses NRF2, which can be attributed to the effect of gonadotrophins and sex steroid hormones, such as follicle-stimulating hormone (FSH), estrogen (E2), and luteinizing hormone (LH) (Liao *et al*., 2012). These hormones can activate NRF2 by inducing ROS levels, which inhibits KEAP1 via oxidation of its multiple cysteine residues (Liao *et al*., 2012). Moreover, NRF2 activation is critical for FSH-induced activation of hypoxia-inducible factor 1 alpha (HIF-1) and vascular endothelial growth factor (VEGF) expression in ovarian cancer, which is critical for tumor angiogenesis (Zhang *et al*., 2013). Thus, these data suggest that NRF2 might also play a key role in the development and progression of hormone-related cancers, such as breast, prostate, and ovarian cancer.

NRF2 INHIBITORS FOR CANCER THERAPY: A REPURPOSING APPROACH

A growing body of evidence suggests hyperactivation of NRF2 in a variety of cancers and its critical role in tumorigenesis and radiochemoresistance; therefore, there is an increasing demand for the development of NRF2 inhibitors for clinical applications (Zhu *et al*., 2016). Although no inhibitors are currently clinically available or under clinical trial, some effective NRF2 inhibitors with potential antitumor efficacy have been reported (Zhu *et al*., 2016). These NRF2 inhibitors include natural compounds extracted from plants such as flavonoids and alkaloids, and novel synthetic compounds, such as ARE expression modulator 1 (AEM1) and ML385 (Bollong *et al*., 2015; Singh *et al*., 2016; Zhu *et al*., 2016). Moreover, some vitamins and commercial drugs developed for other indications have been identified as NRF2 inhibitors, including ascorbic acid (AA), all-trans-retinoic acid (ATRA), antitubercular agents, metformin, and glucocorticoids (GCs). Considering the high risk and time-consuming process of *de novo* anti-cancer drug development, a drug repurposing strategy to develop NRF2 inhibitors could be the first option in the current situation of unmet medical need. Thus, the reported repurposed NRF2 inhibitors are summarized and discussed below.

Ascorbic acid

AA, also known as vitamin C, is a powerful antioxidant and cofactor that participates in diverse enzymatic reactions (Mandl *et al*., 2009; Du *et al*., 2012). AA has been suggested to have anti-cancer properties without cytotoxicity in normal cells by selectively inducing ROS in cancer, but not in normal, cells (Chen *et al*., 2005; Ranzato *et al*., 2011). However, the mechanisms of selective toxicity to cancer cells remain elusive. AA, a reduced form of vitamin C, is taken up by cells through sodium-dependent vitamin C cotransporters (SVCTs), while the oxidized form of vitamin C, dehydroxyascorbate (DHA), is taken up by cells through glucose transporters (GLUTs) (Mandl *et al*., 2009; Du *et al*., 2012). Once inside the cells, DHA is reduced to AA by consuming glutathione (GSH), thioredoxin (TRX), and nicotinamide adenine dinucleotide phosphate (NADPH). Recently, it has been shown that *K-RAS* and *B-RAF* proto-oncogene, serine/threonine kinase (BRAF) mutant colorectal cancer cells are selectively sensitive to AA by overexpressing glucose transporter type 1 (GLUT1), which is responsible for the uptake of DHA (Yun *et al*., 2015). The accumulation of DHA causes depletion of GSH and induction of oxidative stress in the cancer cells, suggesting that AA can be a selective prooxidant in cancer cells conferring cancer specific toxicity. In addition to this mechanism, considering that K-Ras and BRAF oncogenic signals were shown to activate NRF2 as discussed above (DeNicola *et al*., 2011), it would be also plausible to speculate that AA could selectively induce ROS and cytotoxicity in those cancer cells by inhibiting NRF2. Interestingly, a study published more than 10 years ago reported that AA can inhibit NRF2 signaling (Tarumoto *et al*., 2004). The authors showed that the imatinib resistant KCL22/SR leukemia cells have higher NRF2/ARE complex formation ability and NRF2 target expression than the parental imatinib-sensitive KCL22 cell line. AA treatment reduced the binding of NRF2 to ARE, possibly through the inhibition of nuclear translocation of NRF2, and restored imatinib sensitivity. Additionally, another study showed that AA induced the production of too high levels of hydrogen peroxide resulting in the inhibition rather than the activation of NRF2 and heme oxygenase 1 (HO-1) expression in Huh7 liver cancer cells (Wagner *et al*., 2011). Thus, further work is required to determine if NRF2 inhibition is the main mechanism of the anti-cancer effect of AA and if the application of AA could be a promising therapeutic strategy to treat cancer with high NRF2 activity.

Retinoic acid (RA)

Dietary vitamin A is metabolized into biologically active and functionally distinct metabolites called retinoids, which include retinol, retinal, and retinoic acid (RA). Among them, RA is considered the major form that exerts the anti-tumorigenic function of vitamin A, largely by inducing cell differentiation and inhibiting proliferation (Connolly *et al*., 2013). RA functions as a ligand of retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which belong to the type II nuclear receptor family (Duong and Rochette-Egly, 2011). In the absence of RA, RARs and RXRs form heterodimers in the nucleus and recruit transcriptional co-repressors to promoter regions and inhibit transcription. Upon RA binding to the RAR-RXR heterodimer, the co-repressor is replaced with a co-activator in the promoter complex to promote transcriptional activation. Interestingly, RA, particularly all-trans-retinoic acid (ATRA) was shown to inhibit NRF2 through RARα (Wang *et al*., 2007). The RA-RARα complex can bind to NRF2 and interfere with ARE binding of NRF2, without affecting its nuclear translocation. Moreover, in acute myeloid leukemia (AML) and acute promyelocytic leukemia (APL) cells, ATRA was shown to sensitize arsenic trioxide

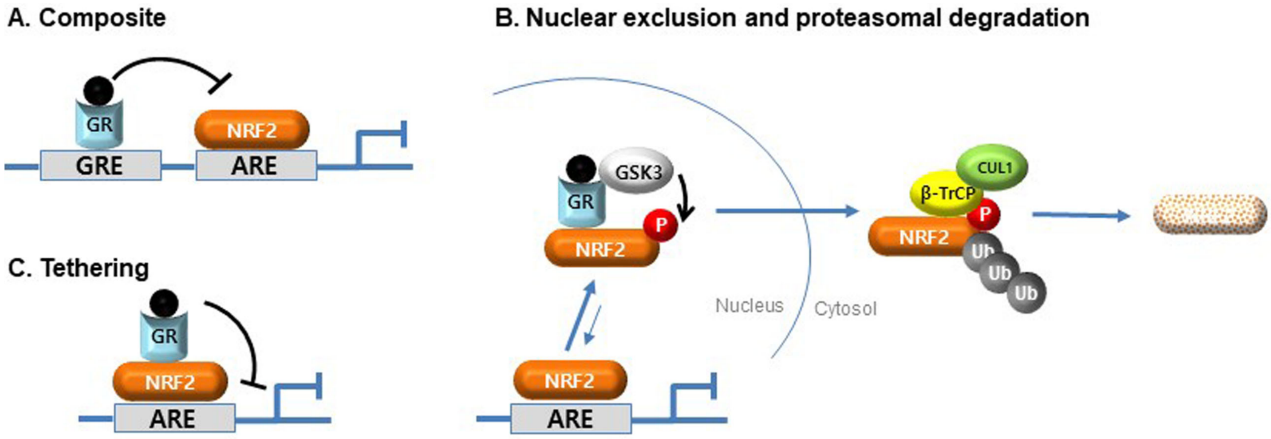

Fig. 3. Proposed mechanisms of nuclear factor E2-related factor 2 (NRF2) inhibition by glucocorticoids (GCs). (A) Composite. (B) Nuclear exclusion and proteasomal degradation. (C) Tethering.

(ATO)-induced apoptosis via inhibition of nuclear translocation of NRF2 (Valenzuela *et al*., 2014) suggesting the potential clinical importance of ATRA in overcoming chemoresistance via inhibition of NRF2. In addition to RARα, RXRα was also shown to inhibit NRF2 through direct interaction with NRF2 via its Neh7 domain (Wang *et al*., 2013). Interestingly, this inhibition of NRF2 does not require RA binding to RXRα or heterodimerization of RXRα with RARα, suggesting that RXR alone is sufficient to inhibit NRF2. Thus, further work is necessary to elucidate the mechanisms underlying the inhibition of NRF2 by RA and RXR and its clinical applications for cancer therapy.

Antitubercular agents: Isoniazid (INH) and Ethionamide (ETH)

INH is the most reliable and commonly used medication for tuberculosis. ETH is a second line drug in tuberculosis therapy, used only in combination with other agents and for drug-resistance tuberculosis. INH and ETH have similar structures and mechanisms of action, and inhibit mycobacterial fatty acid synthesis (enoyl-ACP reductase), which is necessary for cell wall synthesis and repair (Vilcheze and Jacobs, 2014). Moreover, chronic treatment with these drugs induces severe liver injury, leading to acute liver failure as a major undesirable effect (Ramappa and Aithal, 2013). Interestingly, it has been suggested recently that the hepatotoxicity caused by antitubercular drugs is attributed to inhibition of NRF2. In the Hep3B hepatoma cell line, INH prevented nuclear translocation of NRF2 by inhibition of extracellular signal-regulated kinase 1 (ERK1) phosphorylation, which leads to the oxidative stress and apoptosis (Verma *et al*., 2015). In addition, INH effectively inhibited the mRNA expression of NRF2-inducible genes in mouse preadipocyte 3T3-L1 cells (Chen *et al*., 2013). Importantly, via inhibition of NRF2, INH sensitized acute myeloid leukemia (AML) THP1 cells to cytotoxicity by arsenic trioxide (ATO) (Peng *et al*., 2016) suggesting that the inhibition of NRF2 by INH is a novel combination strategy to overcome chemoresistance.

Metformin

Interestingly, NRF2 signaling may also have clinical implications in diabetes management, given that diabetes carries an elevated risk of malignancy (Giovannucci *et al*., 2010) and some common antidiabetic drugs have been suggested as potential NRF2 modulators. Metformin is widely used for the first-line treatment for type 2 diabetes mellitus (Rojas and Gomes, 2013). The anti-diabetic effects of metformin can be attributed, at least in part, to the activation of AMPK by inducing energetic stress caused by inhibition of mitochondrial metabolism. Interestingly, retrospective epidemiological analysis proposed that long-term administration of metformin reduced the incidence of cancer and mortality in diabetic patients (Evans *et al*., 2005; Decensi *et al*., 2010). Moreover, a growing body of evidence supports the anti-tumorigenic effects of metformin, either alone or in combination, in various types of cancer *in vitro* and *in vivo* (Morales and Morris, 2015). Although the involvement of the AMPK-mammalian target of rapamycin complex 1 (mTORC1) axis has been proposed, the mechanisms of metformin's anti-tumor effect remain controversial (Kasznicki *et al*., 2014). Recently, NRF2 inhibition was proposed to mediate the anti-tumor effect of metformin. Metformin reduced *NRF2* mRNA transcription by attenuating the RAF-ERK signaling pathway, but not by activating the AMPK signaling pathway in HepG2, HeLa, and A549 cancer cells (Do *et al*., 2013). A subsequent study by the same group found additional mechanisms by which metformin reduces *NRF2* mRNA transcription through the induction of p53-dependent expression of miR-34a targeting *SIRT1* (sirtuin-1) mRNA, and thereby inhibition of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)-mediated *NRF2* transcription (Do *et al*., 2014). Consequently, metformin enhanced the susceptibility of cancer cells to oxidative stress and tumor necrosis factor superfamily member 10 (TRAIL)-induced apoptosis in a p53-dependent manner, suggesting that p53 status is a critical factor determining the efficacy of combinations comprising metformin. Currently, several clinical trials focusing on the therapeutic effects of metformin as an anti-cancer agent, either alone or in combination with chemotherapeutic drugs, are ongoing (Chae *et al*., 2016).

In contrast, a recent study showed that another class of anti-diabetic drug, dipeptidyl peptidase-4 (DPP-4) inhibitors, might potentially induce NRF2 activation, contributing to acceleration of cancer metastasis (Wang *et al*., 2016). DPP-4 inhibitors reduce blood glucose levels by increasing bioactive incretins, which promote glucose-dependent insulin secretion

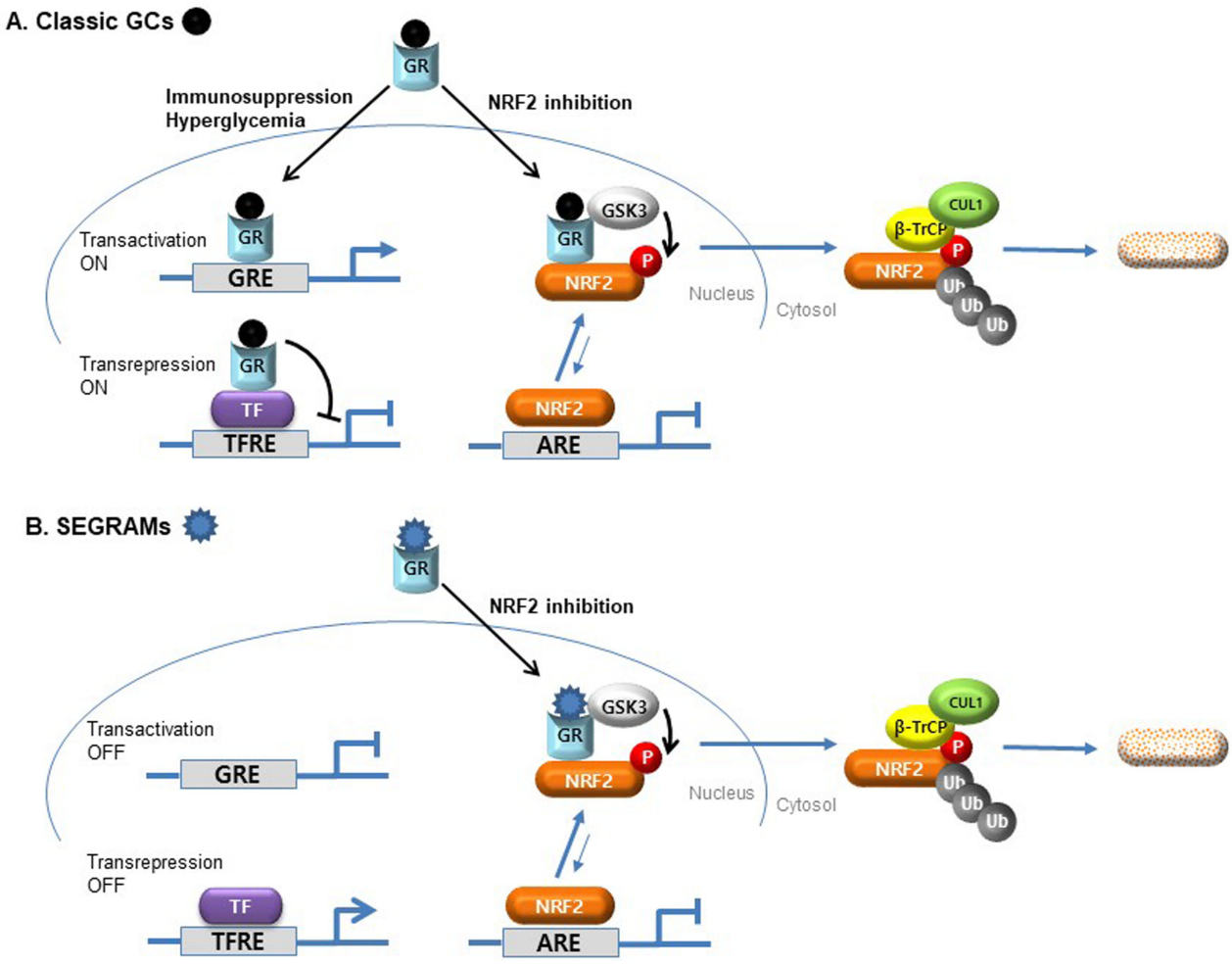

Fig. 4. Principle of the selective inhibition of nuclear factor E2-related factor 2 (NRF2) using selective glucocorticoid receptor agonists and modulators (SEGRAM). (A) The mechanisms of classic GCs on gene expression and NRF2 inhibition. Once binding to GR, GC regulates gene expression via both transactivation and transrepression of diverse genes involved in immunosuppression and hyperglycemia. (B) The proposed mechanisms of SEGRAMs on selective inhibition of NRF2. Although SEGRAMs can bind to GR, it may not be sufficient to induce transactivation or transrepression but sufficient to induce NRF2 inhibition.

and inhibit glucagon secretion from the pancreas to maintain blood glucose homeostasis (Drucker, 2007). However, the mechanisms by which DPP-4 inhibitors induce NRF2 activity are largely unknown. Considering that they have largest prescription volume among the new antidiabetic drug classes (Ahren, 2008; Phung *et al*., 2010; Noh *et al*., 2017), the recent findings regarding their potential NRF2-modulatory effects are of significant importance for patients with diabetes who are chronically exposed to the respective antidiabetic therapy and who are at increased risk of developing malignant complications because of underlying disease. Although the mechanisms of DPP-4 inhibitors' effects on NRF2 activation remain elusive, it might be beneficial to use a DPP-4 inhibitor in combination with metformin in diabetes patients who also have cancer as a comorbidity.

Glucocorticoids (GCs)

Glucocorticoids (GCs), also known as stress hormones, are a class of corticosteroids that play a key role in the regulation of inflammation and metabolism (Kadmiel and Cidlowski, 2013). GCs are synthesized and released from the adrenal cortex upon activation of the hypothalamic-pituitary (HP) axis. The effects of GCs are mediated by binding to the glucocorticoid receptor (GCR), which belongs to the type I nuclear receptor subfamily 3. Upon binding to GCR, the GC-GCR complex translocates to the nucleus to regulate gene expression. Alternatively, the GC-GCR complex can elicit biological effects through direct protein-protein interactions in the cytosol.

The first link between GC and NRF2 came from a study investigating the effect of dexamethasone (DEX), a potent synthetic GC, on the expression of glutathione-S-transferase (GST), a well-known target of NRF2 (Ki *et al*., 2005). The promoter region of GST contains both glucocorticoid response element (GRE) and ARE sequences. In the present study, the DEX-GCR complex inhibited the expression of *GST* through binding to the GRE where it blocked ARE-bound NRF2 activity via silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), suggesting that the inhibition of NRF2 by GCs is confined to certain promoter regions having both GRE and ARE sequences in a composite manner (Fig. 3A). However,

another group showed that cortisol inhibited NRF2 in a ARE-luciferase assay, suggesting that the inhibition of NRF2 by GCs does not require a GRE sequence in the promoter region (Kratschmar *et al*., 2012). Thus, the mechanism by which GCs inhibit NRF2 remains to be elucidated. In addition, the effects of NRF2 inhibition by GCs on cancer was not investigated.

Recently, using a cell-based ARE-luciferase assay, our group reported that unbiased drug repositioning screening identified clobetasol propionate (CP), a GC analog used for various skin disorders, as the most potent NRF2 inhibitor (Choi *et al*., 2017). CP induced both cytosolic accumulation and proteasomal degradation of NRF2 through GCR binding and in a GSK3-b-TrCP dependent manner, suggesting that CP promotes protein-protein interaction between GCR and NRF2 (Fig. 3B). Importantly, CP potently and selectively inhibited anchorage-independent (AI) growth of KEAP1 mutant lung cancer cells, and the cytotoxicity of CP is dependent on the inhibition of NRF2. Notably, CP is 100 times more potent than DEX in the inhibition of NRF2, as well in the AI growth of KEAP1 mutant lung cancer cells. Furthermore, CP, alone or in combination with the mTORC1 inhibitor rapamycin, strongly inhibited the *in vitro* and *in vivo* growth of tumors harboring mutations in *KEAP1* or in both *KEAP1* and *liver kinase B1* (*LKB1*) that are frequently observed in lung cancer.

Consistently, a recent study supported the direct interaction between NRF2 and GCR as the mechanism of NRF2 inhibition by GCs (Alam *et al*., 2017). They showed that GCR was identified as the NRF2 binding protein and that the Neh4/5 transactivation domains of NRF2 interact with GCR. However, DEX inhibited *NRF2* transcriptional activity by promoting GCR recruitment to ARE-bound NRF2 and blocked CBP's interaction with NRF2, suggesting that GCs transrepresses NRF2 by tethering GCR with NRF2, which is a similar mechanism to the inhibition of other transcription factors such as nuclear factor kappa B (NF- κB) and activator protein-1 (AP-1) by GCs (Kassel and Herrlich, 2007) (Fig. 3C).

FUTURE DIRECTIONS

A growing body of evidence has revealed frequent activation of NRF2 via diverse mechanisms in most cancers; therefore, inhibition of NRF2 should be a promising therapeutic strategy to treat cancer. No NRF2 inhibitors are currently available for clinical application; therefore, developing clinically relevant NRF2 inhibitors is highly demanded. Although several NRF2 inhibitors from natural and synthetic compounds, and existing drugs, have been reported, most of them suffer from low potency, non-specificity, and inconsistency in their effects on NRF2 (Menegon *et al*., 2016; Zhu *et al*., 2016). For example, AA, RA, and metformin among the inhibitors have also been reported to activate NRF2 in different setting (Zhu *et al*., 2016). In addition, high (millimolar) concentrations of INH and ETH were used to examine NRF2 inhibition and cytotoxicity *in vitro* (Verma *et al*., 2015; Peng *et al*., 2016). Moreover, the anti-tumorigenic effects of INH and ETH have not been tested *in vivo*. However, consistent results in the inhibitory effect of GCs on NRF2 have been reported (Choi *et al*., 2017). Moreover, GCs, particularly CP, potently inhibited NRF2 and tumor growth *in vivo*, suggesting that only CP is a valid candidate to be developed as an NRF2 inhibitor among clinical compounds (Choi *et al*., 2017). However, the potential limitations of using GCs for cancer therapy are their side effects such as hyperglycemia and immunosuppression. One approach to avoid such potential problems is to develop selective glucocorticoid receptor agonists and modulators (SEGRAM) (Sundahl *et al*., 2016). GCs inhibit NRF2 through the protein-protein interaction between GCR and NRF2, but not through the regulation of transcriptional activity of GCR which is responsible for the effects on metabolism and immune function; therefore, it would be possible to design a GC analog that binds to GCR only to induce the interaction with NRF2 and GSK3, but not sufficient to induce its transcriptional activity (Fig. 4). SEGRAM would be an exciting strategy to develop selective and safe NRF2 inhibitors that warrant further intensive research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by grants from Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2017R1C1B2003162), the National R&D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea (No. 1720070), and the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (No. HI17C0640) to S.-M. Jeon.

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