Molecular Basis of the Keap1-Nrf2 System

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Outline

Introduction

1. *In vivo* validation of Keap1 as a negative regulator of Nrf2
2. Molecular mechanisms of stress sensing by Keap1
3. Two types of Nrf2 inducers as drugs
4. Potential target diseases of Nrf2 inducers
5. Roles Nrf2 plays in oncogenesis
6. Cancer-preventive or -promotive activity of Nrf2 activators in cancer
7. Crosstalk between Nrf2 and other signaling pathway
8. Concluding remarks

Abstract

Nrf2 (NF-E2-related factor 2) is a master regulator of cellular responses against environmental stresses. Nrf2 induces the expression of detoxification and antioxidant enzymes, and Keap1 (Kelch-like ECH-associated protein 1), an adaptor subunit of Cullin 3-based E3 ubiquitin ligase, regulates the Nrf2 activity. Keap1 also acts as a sensor for oxidative and electrophilic stresses. Keap1 retains multiple sensor cysteine residues that detect various stress stimuli. Increasing attention has been paid to the roles that Nrf2 plays in the protection of our body against drug toxicity and stress-induced diseases. On the other hand, Nrf2 is found to promote both oncogenesis and cancer cell resistance against chemotherapeutic drugs. Thus, while Nrf2 acts to protect our body from deleterious stresses, cancer cells hijack the Nrf2 activity to support their malignant growth. Nrf2 has emerged as a new therapeutic target, and both inducers and inhibitors of Nrf2 are awaited. Studies challenging the molecular basis of the Keap1-Nrf2 system functions are now critically important to improve translational studies of the system. Indeed, recent studies identified crosstalk between Nrf2 and other signaling pathways, which provide new insights into the mechanisms by which the Keap1-Nrf2 system serves as a potent regulator of our health and disease. (198 words)

Defense system of our body equips capacity to upregulate the expression levels of cytoprotective enzyme genes. Nrf2 (nuclear factor erythroid 2-related factor 2) is the main player in the inducible expression of our cellular defense enzymes [1, 2]. Nrf2 belongs to the CNC (cap-n-collar) subfamily of basic region-leucine zipper-type transcription factors [3]. Nrf2 dimerizes with one of the small Maf proteins (sMaf) and binds to antioxidant/electrophile response elements (AREs/EpREs) located in the regulatory regions of many defense enzyme genes [1]. Several hundred of Nrf2 target genes have been identified through gene expression profiling analysis and chromatin immunoprecipitation analysis, exploiting the *Nrf2* gene knockout mice as a reference [4]. The Nrf2 target genes encode enzymes involved in the synthesis and conjugation of glutathione, antioxidant enzymes, drug-metabolizing enzymes, transporters, and pentose phosphate pathway enzymes [4-6]. Thus, Nrf2 activates a wide range of cellular defense processes, thereby enhancing the overall capacity of cells to detoxify and eliminate harmful substances. This review will focus on the molecular basis of the Keap1-Nrf2 system function, which is now critically important for the translation study on this system.

1. *In vivo* validation of Keap1 as a negative regulator of Nrf2

Keap1 (Kelch-like ECH-associated protein 1) has been identified as a factor interacting with Neh2 (Nrf2-ECH homology domain 2) degron domain of Nrf2 [2]. Keap1 is an adaptor subunit of a Cullin 3 (Cul3)-based ubiquitin E3 ligase [7]. Under unstressed conditions, Keap1 binds to Nrf2 in the cytoplasm and promotes the ubiquitination and proteasomal degradation of Nrf2. Upon exposure to chemicals (often electrophiles) or reactive oxygen species (ROS), the ubiquitin E3 ligase activity of the Keap1-Cul3 complex declines, and Nrf2 is stabilized. The stabilized Nrf2 accumulates in the nucleus and activates its target genes.

Keap1 function as a negative regulator of Nrf2 has been validated through experiments utilizing mouse genetics. While straightforward Keap1-knockout mice die at the time of weaning due to hyperkeratosis of the upper digestive tract, which leads to feeding problems [8, 9], analyses exploiting knockdown and other hypomorphic lines of mice and mouse embryonic fibroblasts (MEFs) revealed that Nrf2 accumulates in the nucleus and cytoprotective genes are constitutively upregulated in various tissues in the Keap1-deficient mice and in MEFs. The hyperkeratosis and the massive induction of the expression of cytoprotective genes are reversed in *Keap1::Nrf2* compound-knockout

mice, indicating that the phenotype of Keap1-deficient mice is attributable to the constitutive stabilization of Nrf2. These results support the contention that Keap1 is the important negative regulator of Nrf2 *in vivo*.

2. Molecular mechanisms of stress sensing by Keap1

From the beginning of the Keap1-Nrf2 system analyses, this system is linked to a group of biological inducers, collectively named as Nrf2 inducers [11, 12]. It has been found that majority of the chemical Nrf2 inducers are electrophilic and react with nucleophilic thiols, including cysteine sulfhydryl groups [13]. Several mass spectrometry analyses have identified specific patterns of Keap1 cysteine modifications by individual Nrf2 inducing chemicals [14, 15]. The modification of Keap1 cysteine residues results in the inhibition of the ubiquitin E3 ligase activity of the Keap1-Cul3 complex.

The functional significance of these cysteine residues of Keap1 has been examined in experiments exploiting the site-directed mutagenesis [16-18]. The results suggest that the different chemicals that trigger the Keap1-Nrf2 system are associated with distinct patterns of Keap1 cysteine modification. Unique utilization of the sensor cysteine residues leads to the “cysteine code” concept. The cysteine code appears to support the unique Keap1–Nrf2 system function that responds to a diverse array of chemicals and oxidative insults [4].

It has been explored how environmental stresses are transmitted to the gene expression regulation. We have identified Keap1 as a sensor of the electrophiles and ROS, so that the remaining question is how signals detected by Keap1 are transmitted to the Nrf2 activation and induction of cytoprotective gene expression. One unique mechanism proposed for the Keap1-mediated activation of Nrf2 is the “hinge and latch model” [19]. This model has been proposed based on the finding that Keap1 homodimer binds to a single Nrf2 molecule through two distinct binding sites (*i.e.*, DLG and ETGE motifs) within the Neh2 domain of Nrf2. As shown in Figure 1A, both motifs individually bind to a pocket within DC (double glycine repeat and the C-terminal) domain of Keap1 [20]. Binding of high-affinity ETGE motif and low-affinity DLG motif provides a hinge and latch, which facilitates optimal positioning of the lysine residues between the two motifs for ubiquitin conjugation.

Fukutomi and colleagues recently identified that the DLG motif needs to be much more extended (DLG_{ex} motif (Met17-Gln51)) than those assumed by the classical DLG

motif and DIDLID element [21]. The DLGex possesses triple helices, Helix-1 (Leu19-Arg25), Helix-2 (Ile28-Leu30) and Helix-3 (Arg34-Phe37) (Figure 1B). This DLGex structure shows striking difference from the β -hairpin structure of ETGE [22]. The binding modes of DLGex and ETGE to Keap1 are quite distinct from each other. The Keap1-DLGex binding is thermodynamically characterized as both enthalpy- and entropy-driven, and kinetically as fast-on and fast-off. In contrast, the ETGE-Keap1 binding is characterized as purely enthalpy-driven and contains a two-state reaction that leads to stable conformation [21]. These observations support the contention that the DLGex motif serves as a converter transmitting environmental stress to NRF2 induction as the latch-site.

On the other hand, open and closed state of the Keap1-Nrf2 complex was studied by FRET (fluorescence resonance energy transfer) experiments [23]. This study showed that chemical Nrf2 inducers rather strengthen the Keap1-DLGex binding and promote the closed conformation of the Keap1-Nrf2 complex. In this model the latch site becomes too tight and, as a result, Nrf2 is stabilized. Similar observation has been reported through the cancer somatic mutation (super-binder) analyses [24]. While these models do not show immediate consistency (nor discrepancy) with the “hinge & latch” model, the importance of the two-sites binding and the DLG motif participation to the regulation are in common. Further investigations are necessary to verify how these models explain the accumulation of Nrf2.

In this regard, another important observation is that cysteine-151, one of the critical cysteine residues is localized far away from the latch site [25]. Available evidence suggests that modification of this residue may interfere the interaction of Keap1 with Cul3 [26-28].

3. Two types of Nrf2 inducers as drugs

There are substantial interests in developing Nrf2 inducers for therapeutic use. One of the most promising chemical Nrf2 inducers is CDDO (2-Cyano-3,12-dioxooleana-1,9(11)-diene-28-oic acid) triterpenoids derived from the oleanolic acid that has antioxidant and anti-cancer properties [29]. The methyl ester derivative (CDDO-Me) of the triterpenoid compound is a potent inducer of Nrf2. CDDO-Me robustly stimulates the expression of Nrf2-dependent cytoprotective enzyme genes at low nano-molar concentrations. CDDO-Me has been studied in clinical trials, under the generic name Bardoxolone methyl[®], to assess the potential for the treatment of a variety of disorders.

Especially, a phase II clinical trial for chronic kidney disease (BEAM study) showed promising results [30]. Unfortunately, a phase III trial of Bardoxolone (BEACON study) was terminated in October of 2012 due to a higher rate of adverse events [31]. However, CDDO-Me is now under clinical investigation for the treatment of pulmonary hypertension in the USA [<https://clinicaltrials.gov/ct2/show/NCT02036970>] and chronic kidney disease associated with type-2 diabetes in Japan [http://www.kyowa-kirin.com/news_releases/2013/e20131111_01.html]. Another attractive Nrf2 inducer is dimethyl fumarate (Tecfidera[®]), which has been approved for the treatment of relapsing multiple sclerosis by FDA (Food and Drug Administration) [<http://www.fda.gov/Drugs/DrugSafety/ucm424625.htm>]. While the exact mechanisms by which dimethyl fumarate exerts the clinical efficacy remain to be clarified, the effects are believed, at least in part, to be mediated through the Nrf2 activation.

As shown in Figure 2, most of the chemical Nrf2 inducers including the above two are known to interact with cysteine residues of Keap1 and inactivate the Keap1 E3 ligase activity. Since the classic Nrf2 inducers interact with cysteine residues of Keap1 by utilizing their electrophilic nature, these inducers of Nrf2 inherently retain ability to react with intracellular glutathione or thiol of proteins. Therefore, over-dose of the compounds potentially gives rise to toxic effects. In this regard, chemicals directly interrupting the interaction of Keap1 and Nrf2 have been emerging as attractive new inducers of the Nrf2 activity (Figure 2). These non-electrophilic inducers exert their effect without giving electrophilic damages to cells. Two high-throughput screenings searching for small molecular inhibitors of the Keap1-Nrf2 interaction have been reported. One is fluorescence polarization based screening that has identified (SRS)-5 [32], while the other is the homogeneous confocal fluorescence anisotropy assay-based screening that gives rise to Cpd16 [33]. While these non-electrophilic compounds are expected to become safe chemical Nrf2 inducers, further improvements are apparently needed to achieve the low nano-molar efficacy.

4. Potential target diseases of Nrf2 inducers

Nrf2 knockout (*Nrf2*^{-/-}) mice are inherently more susceptible to the oxidative stress-based diseases and drug-induced toxicity. Many lines of experiments demonstrate that Nrf2 activation by pharmacological (*i.e.*, pre-treatment with chemical Nrf2 inducers) or genetic (*i.e.*, the disruption of the *Keap1* gene) approaches significantly reduces cellular damages caused by the oxidative stresses and prevents development and exacerbation of

stress-induced diseases. For example, as shown in Figure 3, 4-nitroquinoline-1-oxide (4NQO)-induced carcinogenesis of the upper aerodigestive tract is nicely prevented by the genetic Nrf2 induction [34]. This cancer preventive effect seem to be due to the high-level expression of Nrf2 and subsequent expression of detoxifying enzymes specific for 4NQO. The detoxification of 4NQO is mostly mediated by glutathione conjugation [35, 36]. Of the Nrf2 target genes, glutamate-cysteine ligase catalytic subunit (Gclc) increases glutathione synthesis and glutathione S-transferase P (Gstp) catalyze formation of 4NQO-glutathione [36]. In addition, thickened stratified squamous epithelium appears to prevent the tissues against the exposure to carcinogens although it has not been examined whether tissue levels of 4NQO are affected by Nrf2 activation (Figure 3).

Another interesting topic emerged as the target disease of Nrf2 inducers is diabetes mellitus. Accumulating lines of evidence demonstrate that the Nrf2 activation brings about the suppression of diabetes mellitus. This anti-diabetic effect of Nrf2 inducers can be explained by two distinct mechanisms. Firstly, the Nrf2 inducers protect pancreatic β -cells against oxidative damages [37]. Secondly, the activation of Nrf2 suppresses the onset and/or progression of insulin resistance in skeletal muscle and liver [38]. These two functions of Nrf2 together lead to the protection against diabetes [39]. In contrast to the above observations of gain-of-Nrf2 function studies, several reports have shown decrease of insulin resistance in Nrf2-knockout mice [40-42]. Although the molecular basis for these bidirectional effects of Nrf2 on insulin resistance (i.e., both gain-of-Nrf2-function and loss-of-Nrf2-function reduce insulin resistance) await further studies, we surmise that the increase of insulin sensitivity brought by gain-of-Nrf2-function may be explained by the suppression of gluconeogenesis [38], Fgf21 elevation [43], AMPK activation [44, 45], and the increase of insulin sensitivity by loss-of-Nrf2-function may be explained by enhancement of insulin signaling due to increased H_2O_2 levels [42].

5. Roles Nrf2 plays in oncogenesis

It has been shown that *Nrf2*^{-/-} mice are susceptible to various carcinogens [34, 38-43]. Showing very good agreement the observations utilizing the *Nrf2*^{-/-} mice, a regulatory single nucleotide polymorphism (rSNP) in human *NRF2* gene upstream promoter region presents intriguing correlation between loss-of-Nrf2-expression and increase-of-carcinogenesis. At 617 bases upstream from the transcription start site, major allele is C/C and minor allele A/A (rs6721961) [52, 53]. The minor A/A

homozygotes exhibit significantly diminished *NRF2* expression in peripheral blood lymphocytes *in vivo* [54] and in reporter co-transfection transactivation assays [53]. When a cohort of lung cancer cases was investigated, the A/A homozygotes show high incidence of non-small cell lung carcinomas, especially in those who had ever smoked [54].

In contrast, analyses of urethane-induced multi-step lung carcinogenesis model exploiting *Nrf2*^{-/-} mice as references give rise to distinct results [55, 56]. Satoh and colleagues have proved that Nrf2 has dual roles in the urethane carcinogenesis. One is to prevent tumor initiation, while the other is to promote malignant progression of the tumors [56]. Consistent with the mouse observation, the lung cancer patients carrying the minor A/A allele at 617 exhibit remarkable survival after surgical operation [57]. These observations demonstrate that Nrf2 plays distinct roles in the initiation and promotion of cancer (Figure 4).

Elevations of NRF2 levels have been identified in various types of human cancers. Somatic mutations, including missense mutations, insertions and deletions, have been identified in both *NRF2* and *KEAP1* genes of various cancers [22, 58-61]. Importantly, these mutations are quite often associated with the stabilization of NRF2. Mutations identified within the *NRF2* gene are clustered within or in the proximity of the DLG and ETGE motifs in the Neh2 domain, which result in the disruption of NRF2-KEAP1 interaction and subsequently the stabilization of NRF2. This natural evidence strongly supports the contention that the integrity of the two-site binding motifs in NRF2 is essential for the NRF2 degradation, and further support the hinge & latch model.

In addition to the somatic mutations in the *KEAP1* and *NRF2* genes, several other mechanisms have been found to provoke NRF2 stabilization. For instance, promoter methylation of the *KEAP1* gene suppresses expression of the gene and results in NRF2 activation [62]. Fumarate hydratase inactivation in renal carcinomas results in the Nrf2 activation via onco-metabolite modification (succination) of the Keap1 cysteine residues by accumulated fumarate although the contribution of Nrf2 activation upon cancer malignancy remains unknown [63, 64]. Transcriptional activation of Nrf2 by Ras oncogenic pathway also contributes to the enhancement of Nrf2 activity in cancer [65]. Various factors can disrupt the normal regulation of the Keap1-Nrf2 pathway, and these are indeed associated with carcinogenesis and/or malignant transformation.

Considering the cytotoxic properties of anti-cancer drugs and the Nrf2 ability to activate the expression of detoxification enzymes, antioxidant enzymes/proteins and

xenobiotic transporters, it seems reasonable to assume that the Nrf2 activation enhances cancer cells resistance to various chemo-radiotherapy [66]. Recently, another line of evidence demonstrating the advantage of constitutive Nrf2 activation for cancer cell proliferation is identified. In rapidly growing cancer cells, Nrf2 acts to redirect glucose and glutamine into the anabolic pathway and supports cell proliferation [67]. Nrf2 also activates the pentose phosphate pathway, indicating that the Nrf2 activation heavily commits to the oncogenic transformation.

6. Nrf2 inducers and carcinogenesis and metastasis

Upon clinical applications of chemical Nrf2 inducers, adverse effects need to be considered. There is a growing concern that Nrf2 inducers may confer resistance to cancer cells that incidentally reside in patients. In this regard, we surmise that accumulating lines of evidence utilizing *Keap1* and *Nrf2* gene-modified mice give rise to impartial view on this issue.

Intriguing observations are that treatment of mice with potent Nrf2 inducers rather decelerates cancer development in certain mouse models of prostate, breast and pancreatic cancers [68-70]. CDDO-Me shows promising preventive and therapeutic activities against certain types of cancers, not only in animal studies but also in clinical trials [71]. The direct anti-tumor activity of CDDO-Me can be explained by inhibition of PI3K (phosphoinositide 3-kinase) and NF- κ B (nuclear factor kappa B) signaling rather than Nrf2 activation [72]. CDDO-Me is also found to abrogate the immune suppressive effect of myeloid-derived suppressor cells (MDSCs) and improved immune responses in cancer patients [73].

It has been shown that Nrf2 exerts protective function against cancer cell metastasis [74]. Upon intravenous injection of Lewis lung carcinoma (3LL) cells, *Nrf2*^{-/-} mice show a significantly increased number of metastatic nodules in the lung, which is associated with aberrant ROS accumulation in MDSCs. In contrast, Nrf2 activation by CDDO-Im reduces the ROS level in MDSCs and effectively prevents the 3LL cancer cell metastasis. This prevention of metastasis is cancelled by myeloid-lineage specific knockout of Nrf2 [75], indicating that the Nrf2 activation in MDSCs prevent cancer cell metastasis.

These wide-ranging observations suggest that the therapeutic benefits for original purpose brought by activating Nrf2 likely exceed the exacerbation risk of Nrf2 activation in pharmacological treatments of various diseases (Figure 5). Furthermore,

Nrf2 inducers may be applicable for the cancer chemotherapy. We surmise that Nrf2 inducers reinforce the cancer chemotherapy if the improvement of microenvironment slows down the growth of cancer cells.

7. Crosstalk between Nrf2 and other signaling pathways

There are a number of reports describing crosstalks between Nrf2 and other signaling pathways. The Keap1-independent degradation pathway mediated by Cul1- β -TrCP (β -transducin repeat-containing protein)-based ubiquitin E3 ligase has been reported [76]. This Cul1- β -TrCP-mediated degradation of Nrf2 is controlled by phosphorylation of Neh6 domain of Nrf2, which can be regulated by GSK-3 [77]. In addition, deletion of PTEN (phosphatase and tensin homolog deleted from chromosome 10) enhances Nrf2 accumulation in Keap1-deficient liver [78], suggesting that sustained activation of PI3K-Akt pathway and inactivation of GSK-3 by the loss of PTEN inhibits Nrf2 degradation via the Cul1- β -TrCP pathway. Interestingly, in long-lived naked mole rats, high basal levels of Nrf2 activity and low expression of Keap1 and β -TrCP are observed [79]. Similarly, loss-of-PTEN-activity correlates well with high Nrf2 protein levels in human endometrioid tumors [80]. While these observations suggest that PTEN/GSK-3/ β -TrCP pathway is an important regulatory axis for the Nrf2 activity, mouse genetic experiments utilizing *Nrf2*, *Keap1*, and *PTEN* knockout lines of mice in combination provide evidence that the PTEN/GSK-3/ β -TrCP pathway exerts effects supplementary for the Keap1 pathway [78].

Another important crosstalk recently identified is between the Keap1-Nrf2 system and cellular autophagy pathway. Autophagy-adaptor protein p62 is found to compete Keap1 binding with Nrf2 by using STGE motif, and through this competition p62 promotes the stabilization of Nrf2 and upregulation of the Nrf2 activity [81]. A key finding here is that phosphorylation of STGE motif in p62 results in strong binding motif to Keap1. The p62 harboring pSTGE (phosphorylated STGE) shows markedly increased binding affinity to Keap1, almost similar to ETGE, and blocks binding of DLG motif [82]. Persistent activation of Nrf2 through accumulation of phosphorylated p62 contributes to the growth of human hepatocellular carcinomas [83].

An intriguing finding in this regard is that, while Nrf2 is predominantly degraded through the proteasome pathway, Keap1 is degraded through the autophagy pathway [84]. The autophagy pathway maintains the integrity and homeostasis of the Keap1-Nrf2 system by governing the Keap1 turnover. Thus, the Keap1-Nrf2 system is

regulated coordinately through the two important cellular proteolysis pathways.

8. Concluding remarks

As summarized above, there have been significant advances in our understanding of the function and regulation of the Keap1-Nrf2 system. One of the important progresses is the clarification of differences in binding mode of DLG and ETGE motifs to Keap1, although precise elucidation of stress response mechanisms needs further studies. Based on the knowledge, chemical Nrf2 inducers that interrupt directly the Keap1-Nrf2 interaction have been emerging as an attractive strategy. Another important progress is the identification of the roles that Nrf2 signaling plays in the oncogenesis. The Nrf2 activity is deeply and differentially involved in the initiation, promotion, and metastasis of cancer cells.

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Figure legends

Figure 1.

The interaction of Keap1 and Nrf2. (A) In response to oxidative and electrophilic stresses, Nrf2 induces the expression of cytoprotective enzyme genes. Under unstressed conditions, Nrf2 is degraded in a Keap1-dependent manner via the ubiquitin (Ub)-proteasome pathway. The Keap1 homodimer binds to single Nrf2 molecule by means of two-site binding utilizing DLGex and ETGE motifs. Both motifs individually bind to a pocket in DC (double glycine repeat and C-terminal) domain of Keap1. Lysine (K) residues that reside between the two motifs are the targets of ubiquitination. Nrf2 inducers inactivate Keap1 via the modification of cysteine residues (Cys), and Nrf2 is stabilized and *de novo* synthesized Nrf2 translocates into the nucleus. Nrf2 heterodimerizes with small Maf proteins (sMaf) and activates target genes through binding to antioxidant response element (ARE), exerting cytoprotective effects against various noxious insults. (B) Phylogenetic alignments of N-terminal Neh2 domain of Nrf2. Conserved amino acid residues are shaded in black. DLGex and ETGE motifs are indicated with pink and red pars, respectively. Three helices in DLGex motif and antiparallel β -sheet in ETGE motif are indicated with black bars and arrows, respectively.

Figure 2.

Two types of Nrf2 chemical inducers. Most of the chemical Nrf2 inducers interact with cysteine residues (Cys) of Keap1 by utilizing electrophilic nature of the molecules and inactivate the Keap1 E3 ligase activity. This process is referred to as the cysteine code. The other type of Nrf2 inducers is non-electrophilic inducers, which interrupt the interaction between Keap1 and Nrf2 directly, and inactivate the Keap1 E3 ligase activity. Non-electrophilic inducers such as Cpd16 and (SRS)-5 likely disrupt only the binding of the DLG motif to Keap1 because binding affinities of these inducers are weaker than binding affinity of ETGE motif.

Figure 3.

Roles the Keap1-Nrf2 system plays in upper aerodigestive tract carcinogenesis by 4NQO. In the absence of Nrf2 (Nrf2 KO), 4NQO dramatically induced carcinogenesis, which is likely due to reduced basal expression of detoxifying enzymes and a relatively

thin stratified epithelium. When Keap1 is knocked down (Keap1 KD), Nrf2 is constitutively activated and 4NQO-induced carcinogenesis is significantly inhibited. In addition to the upregulation of detoxifying enzymes, increase in growth-related metabolic enzyme genes leads to increased progenitor cell growth and thickened stratified squamous epithelium with the keratin layer, which may serve as a mechanical defense.

Figure 4.

Association of NRF2 rSNP and increased risk and reduced malignancy of lung cancers. A/A homozygotes for NRF2 rSNP at 617 exhibit significantly decreased expression of NRF2 and downstream cytoprotective enzyme genes, resulting in the enhanced initiation of carcinogenesis, but reduced malignancy of cancer cells.

Figure 5.

Therapeutic benefits by activating Nrf2 exceed exacerbation risk by activating Nrf2 in cancer cells. Nrf2 inducers potentiate the Nrf2 activity in MDSCs (myeloid-derived suppressor cells) and attenuate the ROS accumulation through activating the antioxidant capacity, resulting in the repression of cancer cell metastasis.

Figure1

Figure 1

Figure 2

Figure 3

Figure4

Figure 4

Figure 5

Graphical Abstract