Roles of NRF2 in Cell Proliferation and Differentiation

Shohei Murakami, Hozumi Motohashi

*Department of Gene Expression Regulation, Institute of Development, Aging and Cancer,
Tohoku University, 4-1 Seiryomachi, Aoba-ku, Sendai, Miyagi, 980-8575, Japan*

CORRESPONDING AUTHOR: Hozumi Motohashi

Hozumi Motohashi (communicating with the Editorial and Production offices)
Department of Gene Expression Regulation, Institute of Development, Aging and
Cancer, Tohoku University,
4-1 Seiryomachi, Aoba-ku, Sendai, Miyagi, 980-8575, Japan
TEL: 81-22-717-8550,
FAX: 81-22-717-8554,
E-MAIL: hozumim@idac.tohoku.ac.jp

Abstract

The KEAP1-NRF2 system plays pivotal roles in defense mechanisms by regulating cellular redox homeostasis. NRF2 is an inducible transcription factor that activates a battery of genes encoding antioxidant proteins and phase II enzymes in response to oxidative stress and electrophilic xenobiotics. The activity of NRF2 is regulated by KEAP1, which promotes the ubiquitination and subsequent degradation of NRF2 under normal conditions and releases the inhibited NRF2 activity upon exposure to the stresses. Though an impressive contribution of the KEAP1-NRF2 system to the protection from exogenous and endogenous electrophilic insults has been well established, a line of evidence has suggested that the KEAP1-NRF2 system has various novel functions, particularly in cell proliferation and differentiation. Because the proliferation and differentiation of diverse cell types are often influenced and modulated by the cellular redox balance, NRF2 has been considered to control these cellular processes by regulating the cellular levels of reactive oxygen species (ROS). In addition, analyses of the genome-wide distribution of NRF2 have identified new sets of NRF2 target genes whose products are involved in cell proliferation and differentiation but not necessarily in the regulation of oxidative stress. Considering the most characteristic features of NRF2 as an inducible transcription factor, a newly emerged concept proposes that the KEAP1-NRF2 system translates environmental stresses into regulatory network signals in cell fate determination. In this review, we introduce the contribution of NRF2 to lineage-specific differentiation, maintenance and differentiation of stem cells, and proliferation of normal and cancer cells, and we discuss how the response to fluctuating environments modulates cell behavior through the KEAP1-NRF2 system.

Keywords: KEAP1-NRF2 system, cell proliferation, differentiation, stem cells

Introduction

The KEAP1-NRF2 system has been known as one of the most critical defense mechanisms against oxidative and electrophilic insults [1]. NRF2 belongs to the Cap'n'collar (CNC) transcription factor family and possesses a unique CNC domain that is linked to the N-terminal side of a highly conserved basic region-leucine zipper (bZIP) motif [2,3]. The basic region is for the DNA recognition, and the leucine zipper is for the dimerization with small MAF (sMAF) proteins [4,5]. Because CNC proteins do not bind to DNA as a monomer, sMAF is an obligatory partner molecule that enables the CNC proteins to bind to DNA and exert their function in the transcriptional regulation [6-8]. The recognition sequence of CNC-sMAF heterodimers is called as Maf recognition element (MARE; TGC TGA ^G/_C TCA GCA), but detailed analyses in recent years have revealed that the CNC-sMAF heterodimers preferentially bind to a MARE-related sequence (CNC-sMAF binding element; GC TGA ^G/_C TCA ^C/_T) rather than to the complete canonical consensus MARE [9-12]. The binding sequence of the NRF2-sMAF heterodimer is designated as an antioxidant responsive element (ARE) or electrophile responsive element (EpRE) (GC nnn ^G/_C TCA) based on its historical background; it was originally identified as a critical *cis*-regulatory element in the transcriptional regulation of antioxidant genes [13-15]. A genome-wide collection of the *cis*-regulatory elements bound by NRF2 is now designated as the “NRF2 cistrome”, and the breadth of the “NRF2 cistrome” is much wider than expected and extends into genes that regulate cell proliferation and differentiation (Table 1).

One of the mechanisms by which NRF2 controls cell proliferation and differentiation is the regulation of reactive oxygen species (ROS) (Fig. 1A). ROS have been recently recognized as one of important signal mediators [16-18]. ROS includes

the initial species formed by reduction of oxygen, like superoxide anion, hydroxyl radical, hydrogen peroxide (H₂O₂), but also encompass other radical and non-radical secondary reactive products generated by reaction with the initial species [19,20]. In this review, we utilize the term “ROS” to indicate all these reactive species that may include experimentally-unidentified reactive products, and instead of identifying which chemical compounds participate in the signal transduction, we describe how ROS are measured in each study. ROS-mediated signaling pathways are heavily dependent on the availability of thioredoxin and glutathione [19,20]. NRF2 increases cellular amounts of reduced thioredoxin and glutathione through upregulating the enzymes involved in their reduction and synthesis, resulting in elimination of ROS in general [21]. This is the way in which NRF2 utilizes ROS as a mediator to transmit the NRF2-activating signals to cellular programs that modify proliferation and/or differentiation.

Other mechanisms are not necessarily related to the regulation of oxidative stress (Fig. 1B). Due to the recent technical advances, the genome-wide distribution of NRF2 has been clarified in several contexts and has led to the identification of novel NRF2 target genes. In this case, NRF2 directly activates the genes that are involved in the regulation of cell fate determination.

Considering that NRF2 is an inducible transcription factor whose activity is regulated by KEAP1 in response to electrophiles, a novel concept emerges in which the KEAP1-NRF2 system translates the oxidative and electrophilic stresses originating from exogenous and endogenous sources into regulatory network signals that influence cell fate determination. In this review, we introduce the NRF2 contribution to the lineage specification, the maintenance and differentiation of stem cells, and the proliferation of normal and cancer cells. We also discuss how the response to fluctuating environments

modulates the processes of cellular development through the KEAP1-NRF2 system.

Regulation of cell differentiation

1) Osteoclastogenesis and osteoblastogenesis

Although ROS were once considered to be toxic pathological compounds that cause cellular damage and dysfunction, recent studies have clarified the physiological roles of ROS as signaling mediators that regulate various cellular functions, including proliferation and differentiation [16-18]. Osteoclasts and osteoblasts are two major players involved in the maintenance of bone homeostasis, where ROS mediate physiological signaling.

Bone homeostasis is maintained based on the balance between formation and resorption mediated by osteoblasts and osteoclasts, respectively, and its imbalance results in pathological conditions such as rheumatoid arthritis and osteoporosis [22]. Osteoclasts are derived from hematopoietic stem cells (HSCs), and osteoblasts differentiate from mesenchymal stem cells (MSCs). The development and differentiation of these two distinct cell types are controlled by a number of cytokines and growth factors. Well-coordinated actions of these factors are essential for the appropriate generation of osteoblasts and osteoclasts and thereby the maintenance of the sophisticated balance between bone formation and bone resorption.

In addition to these classical humoral factors, osteoclast differentiation is modulated depending on the intracellular ROS, particularly H_2O_2 [23,24]. A murine macrophage cell line (RAW264.7 cell) and bone-derived macrophages efficiently differentiate into osteoclasts in response to stimulation with RANK-L, and the intracellular amounts of ROS become elevated as they differentiate. Interestingly,

attenuated NRF2 activity increases the accumulation of ROS measured by dye oxidation protocol such as dichlorodihydrofluorescein diacetate (H₂DCFDA) along with decrease in reduced glutathione, and enhances osteoclast differentiation [25,26] (Fig. 1A (i)). Treatment of osteoclast precursor cells with N-acetylcysteine (NAC), an antioxidant agent, inhibits osteoclast differentiation, which indicates that increased ROS facilitate osteoclastogenesis in the absence of NRF2.

While NRF2 activation inhibits osteoclast differentiation by reducing the intracellular ROS, osteoblast differentiation is also inhibited by NRF2, but this is not dependent on redox regulation in this case. It has been shown that NRF2 directly associates with RUNX2, which is a key regulator of the osteoblast differentiation [27,28], and suppresses its transcriptional activity [29]. Interestingly, the interaction between NRF2 and RUNX2 is likely to impair the differentiation of chondrocytes as well. Similar to osteoblasts, chondrocytes are derived from MSCs, and RUNX2 plays a pivotal role in chondrogenesis [30]. NRF2 overexpression in the ATDC5 pre-chondrogenic cell line inhibits chondrocyte maturation [31].

As mentioned above, NRF2 inhibits the differentiation of both osteoclasts and osteoblasts, which raises a question of how NRF2 contributes to bone homeostasis *in vivo*. There is a study answering to the question by demonstrating that the inhibitory effect of NRF2 is dominant in the osteoblast differentiation *in vivo* [32]. Compared with wild-type mice, *Nrf2*-deficient mice exhibit an increase in bone mass due to a larger number of osteoblasts. Whereas NRF2 disruption enhances osteoclastogenesis *in vitro* under RANK-L stimulation, no functional enhancement of osteoclasts is apparent in *Nrf2*-deficient mice. This discrepancy is due to osteoprotegerin (OPG), which is a decoy receptor of RANK-L that is secreted from osteoblasts *in vivo*. Osteoclastogenesis is

accomplished through the fine balance between RANK-L and OPG, and a predominance of OPG inhibits osteoclast differentiation [33,34]. The study suggests that NRF2 negatively regulates the expression of OPG in osteoblasts through direct binding to AREs in the promoter region of the OPG gene, although NRF2 usually serves as a transcription activator [32]. Therefore, it is likely that NRF2 deficiency enhances OPG expression and thereby suppresses osteoclastogenesis *in vivo*.

In contrast, under stressed conditions, the impact of NRF2 deficiency is more obvious in osteoclasts than osteoblasts. Ionizing radiation (IR) generates oxidative stress, which is implicated as a cause of IR-induced osteoporotic phenotypes [35,36]. The bone loss initiated by IR is augmented in *Nrf2*-deficient mice, which is accompanied by a higher number of osteoclasts than wild-type mice [37]. Interestingly, *Nrf2*-deficient osteoblasts increase the RANK-L expression after IR, presumably resulting in osteoclast expansion.

Overall, the impacts of the NRF2 deficiency on bone homeostasis, especially the behavior of osteoclasts and osteoblasts, seem to be context dependent. Environmental stress, *i.e.*, irradiation, changes the contribution of NRF2 to the maintenance of bone homeostasis.

2) Adipogenesis and obesity

Adipocyte differentiation is another well-studied system in the context of the association between NRF2-dependent transcription networks and cell maturation, and it is closely associated with the pathogenesis of diabetes. Two factors responsible for adipogenesis are directly regulated by NRF2 [38,39] (Fig. 1B (i)). One is CCAAT/enhancer-binding protein β (CEBP β), which is transiently expressed at the onset of adipocyte

differentiation and initiates the differentiation programs. The other is peroxisome proliferator-activated receptor γ (PPAR γ), which orchestrates the differentiation process downstream of CEBP β [40]. NRF2 regulates these two genes through binding to the AREs located in each promoter region, and its defect suppresses adipogenesis *in vitro* [38,39]. Supporting this *in vitro* observation, either global or adipocyte-specific disruption of *Nrf2* gene reduces white adipose tissue mass in *ob/ob* mice, which is an obese mouse model characterized by leptin deficiency [41]. The *ob/ob* mice with adipocyte-specific disruption of *Nrf2* exhibit aggravation of insulin resistance, hyperglycemia and hypertriglyceridemia, which may be associated with the limited deposition of lipids in the *Nrf2*-deficient adipose tissues. These studies support the notion that NRF2 promotes adipocyte development.

Whereas NRF2 deficiency impairs adipocyte development, NRF2 activation also decreases adipose tissue mass and lipid accumulation in the *ob/ob* mice and limits the induction of adipocyte differentiation from embryonic fibroblasts (MEF) *in vitro* [42]. Similarly, previous studies have shown that NRF2 inhibits adipocyte differentiation by directly activating the transcription of the aryl hydrocarbon receptor (*AhR*) gene or the retinoid X receptor α (*Rxra*) gene, which are involved in lipid metabolism [43,44] (Fig. 1B (ii)). AHR is decreasingly expressed during adipogenesis, and the forced expression of AHR inhibits the differentiation of MEFs into adipocytes [43]. Thus, AHR is one of the candidates downstream effectors of NRF2 in adipogenesis inhibition. RXR α is another candidate and exerts repressive effects on adipogenesis by changing the RXR α /RXR β ratio that modulates PPAR γ activity [45]. Therefore, the NRF2-mediated upregulation of the *Rxra* gene appears to perturb the fine tuning of the RXR α /RXR β ratio during adipogenesis.

Similar to the NRF2 functions in bone homeostasis, an *in vitro* differentiation system examining cell-autonomous roles of NRF2 has shown the context-dependent contribution of NRF2 to adipogenesis. In addition to the cell-autonomous effects of the NRF2 activity, adipocyte development and lipid deposition in adipocytes are heavily influenced by the systemic energy metabolism, involving skeletal muscles, liver and pancreas. In particular, the balance between energy intake and expenditure is one of the most critical determinants of adipose tissue mass. A recent investigation has revealed that systemic activation of NRF2 due to *Keap1* gene knockdown improves insulin resistance and prevents obesity induced by a high-fat diet [46]. Another study demonstrated that selective activation of NRF2 in pancreatic β cells robustly preserves β cells in the iNOS-Tg diabetes mouse model and mitigates the progression of diabetes [47]. Thus, these cell-autonomous and non-cell-autonomous factors would be integrated into the observable NRF2 functions in adipogenesis *in vivo*.

3) Spermatogenesis and other tissue development

It is known that spermatozoa are highly susceptible to oxidative stress and need to be protected by sufficient amounts of antioxidants in the reproductive tract [48,49]. Consistent with this notion, spermatogenesis is closely associated with NRF2 activity. *Nrf2*-deficient male mice exhibit decreased fertility due to accumulating damage to germ cells with increasing age [50]. Testicular and epididymal glutathione and antioxidant enzyme activities are significantly lower in *Nrf2*-deficient mice than in wild-type mice. Conversely, lipid peroxidation products are elevated in *Nrf2*-deficient testes and epididymides. These results indicate that NRF2 prevents oxidative stress in spermatozoa and supports spermatogenesis.

Intriguingly, single nucleotide polymorphisms (SNPs) in the human *NRF2* gene promoter region have been found to associate with idiopathic oligoasthenozoospermia [51]. One of the SNPs, rs6721961, corresponds to a critical determinant of the transcription levels of the *NRF2* gene, and the decreased NRF2 expression due to the SNP is significantly associated with a higher frequency of lung cancers in smokers [52]. Therefore, it is plausible that the low NRF2 activity that results from the SNPs causes spermatozoa to be highly susceptible to oxidative damage and consequently results in defective spermatogenesis.

In addition to cell differentiation, the NRF2-mediated modification of tissue development has been observed in lung development and epidermal barrier formation [53,54]. NRF2 prevents neonatal pulmonary cells from hyperoxic cell death [53]. NRF2 also compensates for defective epidermal barrier formation in the absence of loricrin, a glycine-/serine-rich structural protein in a cornified envelope [54]. In both cases, NRF2 is vital for tissue development in the presence of disruptive factors originating from exogenous or endogenous sources, whereas NRF2 is dispensable under non-stressed conditions. In summary, NRF2 guarantees a normal process of tissue development by maintaining the redox homeostasis in response to environmental fluctuations and also transmits the environmental signals to the molecular machinery that regulates the differentiation process by directly controlling tissue-specific regulators of differentiation.

Contribution to stem cell maintenance and cell fate determination

1) Interaction between NRF2 activity and ROS in stem cells

Stem cells are characterized by self-renewal ability and multipotency, and most of them

are often maintained in quiescence under normal conditions. The dormancy is beneficial for stem cells to avoid unnecessary DNA replication and eventual exhaustion by reducing the chance of DNA damage and mutations [55-58]. Recently, ROS have been suggested as one of the important regulators of stem cell self-renewal [59]. However, the roles and functions of ROS in stem cells are diverse and presumably context dependent [60-62]. In some cases, stem cells are stimulated to proliferate by increased ROS including H₂O₂ that are a major origin of ROS signaling pathways [60,61], but in other cases, ROS accumulation deteriorates stem cell functions and maintenance, which can be rescued by NAC treatment [62-64]. Thus, it is not surprising that observable contributions of the KEAP1-NRF2 system to stem cell activity also vary from study to study depending on experimental systems.

It has been reported that CncC, which is a *Drosophila* homolog of NRF2, is constitutively active in intestinal stem cells (ISCs) of *Drosophila* and maintains ISCs quiescent with low ROS concentration [65] (Fig. 1A (ii)). Interestingly, for the ISC proliferation that is induced by an increase in ROS accumulation, the KEAP1-mediated inhibition of CncC is required. In this experimental setting, it is unclear what reactive components are the substantial mediators, because ROS were measured by dye oxidation protocol using dihydroethidium (DHE), which has been suggested to have several limitations [66,67]. However, overexpression of *gclc*, the rate-limiting enzyme for glutathione synthesis, and *jafrac1*, a thioredoxin peroxidase, leads to decrease in the KEAP1-mediated ISC proliferation along with reduced levels of oxidative stress. Therefore, it is interpreted that ROS are a downstream effector of the KEAP1-NRF2 system, and that the ROS elevation caused by the CncC repression drives the ISC proliferation.

In other cases, ROS are not necessarily a major downstream effector of NRF2 in stem cell proliferation. In bronchial epithelia, airway basal stem cells (ABSCs) play important roles in the maintenance of airway homeostasis [68,69]. When the tracheobronchial epithelium is injured by environmental hazards or stress, ABSCs are driven into proliferation and differentiation to replenish epithelial cells. There is a positive correlation between the ROS concentration and the ABSC proliferation [70]. Exposure of ABSCs to exogenous H₂O₂ increases the intracellular concentration of ROS, which is defined by H₂DCFDA, DHE, and Mito Sox, and accelerates the ABSC proliferation, whereas the NAC treatment takes the opposite effect. The NRF2 activity is enhanced as the ROS increase, implying that NRF2 may be activated in response to the increase in ROS [70]. Importantly, downstream of the ROS-mediated NRF2 activation, NOTCH1 is responsible for the proliferation of ABSCs (Fig. 1B (iii)). The introduction of the intracellular domain of NOTCH1 almost completely rescues the impaired ABSC proliferation in the absence of NRF2. Therefore, for ABSC proliferation, NRF2 conveys the ROS signals to the NOTCH1 pathway.

2) NRF2-dependent gene regulation in stem cells and progenitor cells

There are some situations in which NRF2 regulates the maintenance and differentiation of stem/progenitor cells through mechanisms that are unrelated to intracellular ROS control. One example is found in the control of self-renewal and pluripotency of human embryonic stem cells (hESCs) by NRF2 [71]. NRF2 is highly expressed in undifferentiated hESCs compared with differentiated cells, implying the functional significance of NRF2 in the pluripotent status of hESCs. Indeed, a reduction of NRF2 activity enhances the differentiation of hESCs, and the maintenance of pluripotency is

inhibited by *Nrf2* knockdown. A decrease in proteasome activity induced by the NRF2 knockdown has been suggested to be an underlying mechanism of the impairment of pluripotency because the sufficient levels of proteasome activity is essential for the maintenance of pluripotency. Among the genes required for appropriate proteasome activity, POMP, a chaperone for the assembly of proteasomal subunits, is one of the candidates that fall under the control of NRF2. Taken together, NRF2 is at least partly responsible for the maintenance of hESCs through modulation of proteasome activity.

Another example is found in satellite cells (SCs) that form a resident population of stem/progenitor cells in skeletal muscles. SCs are maintained in a quiescent state outside the cell cycle under normal conditions. Upon the skeletal muscle injury, they begin to proliferate and differentiate, which is an essential process of muscle repair [72,73]. The proliferation and differentiation of SCs are induced by myogenic regulatory factors, MyoD and myogenin, respectively [72,73]. During muscle regeneration, NRF2 supports the efficient proliferation of SCs by upregulating MyoD and suppressing myogenin expression [74]. Consequently, *Nrf2* deficiency delays muscle regeneration after the injury. Because MyoD has a well-conserved functional ARE in its promoter region, it appears to be a direct target of NRF2 in the activation of dormant SCs during the injured muscle repair (Fig. 1B (iv)).

Neuronal progenitor cells (NPCs) still provide another example in which NRF2-dependent gene regulation accounts for its regenerative activity. Increased NRF2 activity enhances both proliferation and differentiation of NPCs, and functional impairment of NRF2 retards compensatory neurogenesis in a global brain ischemia model [75]. Consistent with this observation, NRF2 activation potentiates retinoic acid-induced neuronal differentiation of a neuroblastoma cell line, SH-SY5Y cells,

whereas the NRF2 activation itself is insufficient to stimulate the differentiation [76].

We can deduce from these examples that NRF2 plays distinct roles in pluripotent stem cells and in tissue stem cells. In hESCs, NRF2 inhibits differentiation but contributes to the maintenance of pluripotency [71]. In SCs and NPCs, which are categorized into tissue stem cells, NRF2 enhances proliferation and/or differentiation for tissue regeneration [74,75]. In the following section, we focus on NRF2 functions in hematopoietic stem cells (HSCs), which are one of the best-characterized tissue stem cells.

3) Roles of NRF2 in hematopoietic stem cells (HSCs)

Due to the well-established transplantation technique, the characterization of HSCs is much more advanced than that of other tissue stem cells. Most HSCs are maintained in a quiescent state and hardly contribute to daily hematopoiesis [77,78]. However, under stressed conditions, *e.g.*, during inflammation or after bone marrow transplantation, HSCs proliferate, increase in number and produce progenitor cells and, subsequently, mature cells [77-80]. Several studies have shown that HSCs are vulnerable to oxidative stress [63,64,81]; thus, to escape from oxidative stress, HSCs are localized in special environments, called “niches”, where the oxygen levels are relatively low [62,82]. For example, when the genes essential for HSC function and maintenance are deleted in HSCs, such as FOXOs and TSC1, accumulation of ROS is observed [63,81,83]. While it is obscure what reactive species are responsible for the HSC exhaustion, ROS are considered as major causes for HSC exhaustion, based on the fact that the HSC impairment is partially restored by NAC treatment [63,81,83].

The results of these reports lead to the expectation that the KEAP1-NRF2 system

engages in the control of HSCs. Indeed, *Nrf2* deficiency attenuates the reconstitution capacity of HSCs after the transplantation, indicating that NRF2 is required for the HSC maintenance [84,85]. Of note, this effect of NRF2 on the HSC maintenance is independent of cellular ROS concentration [84]. One study suggests that NRF2 directly binds to ARE in the promoter region of *Cxcr4* [85], which is indispensable for the maintenance of the quiescent HSC pool [86] (Fig. 1B (iv)). The reduced CXCR4 signaling disturbs the maintenance of HSCs in the absence of NRF2 [85].

In addition to the *Nrf2*-deficient model, *Keap1*-deficient HSCs, in which NRF2 is constitutively activated, were examined to clarify the contribution of NRF2 activation to the stem cell function [87]. *Keap1* deficiency in HSCs mitigates irradiation-induced myelosuppression and promotes the early reconstitution of bone marrow cells after transplantation. The enhancement of HSC activity is attributed to the upregulation of the NOTCH1 pathway, as observed in ABSCs (Fig. 1B (iii)). These results clarify a benefit of NRF2 activation in HSCs for short-term reconstitution. However, further analyses are required to understand the contribution of NRF2 to the long-term reconstitution ability of HSCs.

The KEAP1-NRF2 system also contributes to the differentiation as well as maintenance of HSCs. Recently, we found that NRF2 activation skews the differentiation potential of HSCs and facilitates granulocyte/monocyte production at the expense of erythroid and lymphoid cells [88]. In *Keap1*-deficient hematopoietic cells, the expression levels of genes that are important for lineage specification are already altered at the stage of HSCs from erythroid and lymphoid to myeloid dominance, and the skewing is restored by simultaneous disruption of *Nrf2* on the *Keap1*-null background. Therefore, NRF2 activation in HSCs drives the myeloid differentiation and represses

erythroid and lymphoid differentiation.

The effect of NRF2 on hematopoietic differentiation has been demonstrated not only in normal HSCs but also in acute myeloid leukemic (AML) cells [89]. It has been shown that NRF2 activation enhances the 1,25D (1 α ,25-dihydroxyvitamin D₃)-induced differentiation of AML cells by increasing the activity of activator protein-1 (AP-1) and the subsequent elevation of vitamin D receptor and RXR α expression. The activation of NRF2, which drives the maturation of leukemic cells beyond the differentiation block, might be a promising strategy for differentiation therapy of leukemia.

4) Roles of NRF2 in other hematopoietic cell lineages

Given that NRF2 is expressed throughout the differentiation stages of hematopoietic cells, NRF2 contributes broadly to the differentiation and maturation of more differentiated cells, such as erythrocytes and megakaryocytes [90-92]. NRF2 was initially identified as one of the regulators of erythroid-specific genes, such as the β -globin gene [93], but early studies concluded that NRF2 was not essential for erythroid production because *Nrf2*-deficient mice did not display any apparent defects in erythropoiesis [94-96]. However, this conclusion has been recently challenged by detailed analyses of erythropoiesis in *Nrf2*-deficient mice. One study demonstrated that aged *Nrf2*-deficient mice exhibit a decreased red blood cell count as a result of immune-mediated hemolysis [90]. *Nrf2*-deficient erythrocytes are morphologically abnormal and are more sensitive to H₂O₂-induced hemolysis than are wild-type erythrocytes. Due to an increased level of oxidative damage, an increased number of erythrocytes are bound by IgG in *Nrf2*-deficient mice, which results in the immune-mediated hemolysis. In support of this study, *Nrf2* has been found to be essential for the protection of erythrocytes from

hemolytic anemia by regulating redox homeostasis in erythrocytes [91]. When the synthesis of antioxidant selenoproteins is defective, NRF2 makes a larger contribution to erythrocyte protection than under normal conditions and compensates for the impaired ROS scavenging activity. Thus, NRF2 plays a critical role in the maintenance of redox homeostasis in erythrocytes to prevent hemolytic anemia, while NRF2 activation limits the production of erythrocytes [88].

In megakaryopoiesis, NRF2 is involved in the regulation of megakaryoblast proliferation and megakaryocyte maturation by modulating the activity of NF-E2 p45 [92], which is a key regulator of megakaryopoiesis and platelet production [12,97,98]. NRF2 and NF-E2 p45 both belong to the CNC transcription factor family and share the CNC-sMAF binding elements [3,4,99]. In immature megakaryoblasts, NRF2 is dominant and promotes their proliferation, whereas NF-E2 p45 dominates over NRF2 and outcompetes NRF2 for the binding sites in mature megakaryocytes [92]. Because the transcriptional activation ability of NF-E2 p45 is weaker than that of NRF2, occupancy of the AREs by NF-E2 p45 limits the expression of antioxidant proteins and consequently increases the intracellular concentration of ROS, which is critical for the terminal maturation of megakaryocytes and platelet production. According to the fact that inhibition of NADPH oxidases impairs the maturation of megakaryocytes, particularly polyploidisation, superoxide anions produced by NADPH oxidases are one of essential components for megakaryocyte differentiation [100]. Megakaryocytes provide a prototype demonstrating the significance of functional balance among CNC family proteins in transcriptional regulation via the CNC-sMAF binding elements.

Other CNC family members, including NRF1, NRF3, BACH1 and BACH2, are also considered to share the DNA binding site in principle [101-104]. Competitive

relations have been reported between NRF2 and BACH1 in the regulation of *Ho-1* gene in various cell lineages [104,105] and between NRF2 and NRF1 in the regulation of xCT gene in liver [106]. In addition, a recent report has suggested an intriguing possibility that the balance between NRF2 and BACH2 regulates the myeloid and lymphoid differentiation [107]. BACH2 is a key regulator of B cell development and maturation [108], and its deficiency enhances myeloid-specific gene expression and skews the differentiation of progenitor cells toward the myeloid lineage [107]. Considering that NRF2 activation promotes myeloid differentiation at the expense of lymphoid differentiation [88], this lineage skewing in *Bach2*-deficient mice might be mediated by NRF2.

5) Cancer stem cells and Nrf2 activation

Cancer stem cells (CSCs) are considered to be a rare subpopulation that exhibits high resistance to radiotherapy and chemotherapy [109,110]. Their therapeutic resistance is mostly attributable to cell cycle quiescence and to the elevated expression of antioxidant proteins. Because NRF2 is a master regulator of genes encoding antioxidant proteins, detoxification enzymes and drug transporters, NRF2 activity is likely to account for the features of CSCs.

Recent cancer genome analyses have identified somatic mutations in the KEAP1-NRF2 pathway in various human cancers, especially in lung, bladder, and head and neck [111-113]. In many cases, the mutations in *KEAP1* and *NRF2* genes result in constitutive activation of NRF2 and elevated expression of cytoprotective genes [114-116]. Higher and lower NRF2 activity is associated with a worse and better prognosis of patients, respectively [116-120], which suggests critical roles for NRF2

target gene products in the acquisition of tumorigenicity and therapeutic resistance. Among numerous studies on NRF2 activation in cancers, some reports have elaborated on the NRF2 functions in CSCs.

In glioma stem cells (GSCs), which are one of the best-studied CSCs [121,122], NRF2 has been found to be necessary for their self-renewal capacity [123]. Consistently, NRF2 expression is higher in GSCs than non-stem-like fractions [124]. Another report demonstrated that the induction of an NRF2-dependent transcriptional network supports the survival and self-renewal of GSCs when they are challenged with anticancer agents [125].

Significant roles of NRF2 have been also described from the viewpoint of de-differentiation of cancer cells. The de-differentiation of cancers, which is often mediated by epithelial-to-mesenchymal transition, confers drug resistance and CSC-like properties. NRF2 has been shown to be activated in the de-differentiated cells via phosphorylation by ER membrane kinase PERK, eliciting the expression of multidrug resistant genes [126].

Overall, NRF2 contributes to the survival, self-renewal and acquisition of therapeutic resistance of CSCs, resulting in the malignant behavior of cancers and poor clinical outcomes.

Contribution to cell proliferation

1) Interaction with the PI3K-AKT pathway

Though the beneficial effects of NRF2 activation in tissue regeneration have been explained here so far through the NRF2 functions in tissue stem cells, NRF2 also promotes the proliferation of differentiated cells. The liver has high regenerative activity,

and liver regeneration provides an ideal experimental system for examining the NRF2 contribution to reactive cell proliferation. *Nrf2*-deficient mice exhibit a significant delay in liver regeneration after partial hepatectomy, with a decreased number of proliferating hepatocytes and an increased number of apoptotic hepatocytes [127]. The amount of ROS, determined by H₂DCFDA and DHE, is also higher in the *Nrf2*-deficient hepatocytes than in wild-type control. Given that tumor necrosis factor α -induced ROS production provokes apoptosis in hepatocytes [128], the NRF2 deficiency seems to cause the ROS accumulation, which results in the increased apoptotic hepatocytes in the regenerating liver. Insufficient NOTCH1 signaling is proposed as another cause of the delayed liver regeneration in *Nrf2*-deficient mice because *Notch1* is a direct target of NRF2 [129] (Fig. 1B (iii)). Supporting this concept, the forced expression of the intracellular domain of NOTCH1 rescues the delayed liver regeneration in *Nrf2*-deficient mice. Conversely, NRF2 activation enhances reactive proliferation in hepatocytes after portal vein branch ligation [130].

During liver regeneration due to partial hepatectomy, the activation of the PI3 kinase (PI3K)-AKT signaling pathway was attenuated in *Nrf2*-deficient mice, suggesting a novel role of NRF2 in the regulation of growth factor signaling [127]. We employed an alternative approach and showed a reciprocal activation loop between NRF2 and the PI3K-AKT signaling pathway [131,132]. Liver-specific disruption of *Keap1* stabilizes NRF2, and liver-specific *Pten* activates the PI3K-AKT pathway and increases the phosphorylation level of GSK3. Notably, liver-specific *Keap1* and *Pten* double disruption results in the massive accumulation of NRF2 with robust activation of NRF2 target genes and a remarkable increase in the phosphorylation of AKT and GSK3 [132]. The increased phosphorylation of AKT and GSK3 is both canceled by the additional

disruption of *Nrf2*, indicating that NRF2 activation is responsible for the augmentation of PI3K-AKT signaling in the double mutant liver. Elucidation of the mechanisms underlying the potentiation of PI3K-AKT signaling by NRF2 requires further investigation.

In contrast, the enhancement of NRF2 activity by active PI3K-AKT signaling is explained by the inactivation of a KEAP1-independent degradation system of NRF2. Recent studies have clarified a novel mechanism of NRF2 degradation, which is mediated by CUL1- β TRCP E3 ubiquitin ligase in cooperation with GSK3 [133,134]. Once phosphorylated by GSK3, NRF2 is trapped and ubiquitinated by CUL1- β TRCP complex and is finally degraded. In the sustained activation of PI3K-AKT pathway, GSK3 is phosphorylated and inactivated, which leads to the shutting off of the CUL1- β TRCP-mediated degradation of NRF2. Importantly, the inactivation of the KEAP1-independent degradation takes effect in the absence of KEAP1 [132], suggesting that the NRF2 that escapes from the KEAP1-dependent degradation becomes a substrate of the KEAP1-independent degradation system.

Massive accumulation of NRF2 in *Keap1::Pten* double deficient liver causes remarkable hepatomegaly with an aberrant increase in tubular structures composed of cholangiocytes [132]. An interesting connotation that has emerged from this result is that NRF2 not only promotes proliferation of liver cells but also influences the lineage commitment between hepatocytes and cholangiocytes. Taken together, it is plausible that NRF2 establishes a crosstalk with the PI3K-AKT pathway to regulate cell growth and fate decisions in various tissues.

2) Regulation of cell cycle-related factors

ChIP-sequencing analyses for the genome-wide distribution of NRF2 in combination with gene expression analyses have identified novel sets of NRF2 target genes [11,44,135]. For instance, proliferation-associated genes, including *Bmpr1a*, *Igf1*, and *Jag1*, have been identified as direct targets of NRF2 [135]. The list of NRF2 target genes includes *Cdkn1a* (*p21*) and *Cdkn2b* (*p15*), which are inhibitory cell-cycle regulators. These genes may be related to stress-induced cell cycle arrest in the NRF2-mediated response to oxidative stress.

It has been described that NRF2 is required for the transition from G2 phase to M phase in the cell cycle, based on the observation that functional impairment of NRF2 compromises or delays M-phase entry [136,137]. The G2/M transition is driven by the activation of Cdk1/Cyclin B1 pathway, and the Cdk1/Cyclin B1 complex activity is diminished by the phosphorylation of Cdk1 by Wee1 kinase [138,139]. The accumulation of Wee1 and hyperphosphorylation of Cdk1 is observed in *Nrf2*-deficient hepatocytes during liver regeneration, which presumably causes G2/M arrest [136]. Another study proposes that glutathione is a substantial modifier of cell cycle status downstream of NRF2 because the G2/M arrest caused by the NRF2 deficiency is rescued by glutathione supplementation, but interestingly not by NAC [137]. These results suggest that NRF2 activity is closely associated with cell cycle regulation.

3) Regulation of cellular metabolism

It has been well established that higher activity of NRF2 in cancer tissues is strongly correlated with poor prognosis for patients [116-120]. This is not difficult to understand, considering that NRF2 confers resistance against chemotherapy and radiotherapy by coordinately activating cytoprotective genes. In addition, one study has provided a new

concept of the roles of NRF2 in cancer malignancy by demonstrating that KRAS-mediated carcinogenesis and cancer progression is inhibited in *Nrf2*-null mice [140]. Their finding implies that NRF2 is required in the early phase of carcinogenesis and cancer progression and is involved in aspects other than simply conferring therapeutic resistance.

Highly proliferative cells, such as cancer cells, possess fundamentally different metabolic activities from quiescent cells [141]. Proliferating cells require abundant nutrients for the synthesis of cellular components including lipids, proteins and nucleic acids. We have recently found that the activity of NRF2 is augmented under sustained activation of the PI3K-AKT pathway, which enables NRF2 to activate metabolic genes involved in the glucose and glutamine metabolism and contribute to metabolic reprogramming for cell proliferation [131]. In particular, NRF2 activates the four key enzyme genes of the pentose phosphate pathway, *G6PD*, *PGD*, *TKT* and *TALDO1*. Direct regulation of these metabolic genes by NRF2 has been confirmed in an independent ChIP-sequencing analysis with an anti-NRF2 antibody [11]. In addition, two types of microRNAs, miR-1 and miR-206, have been shown to regulate these genes under the control of NRF2 [142].

The activation of NRF2 indeed alters metabolite flux, shunting glycolytic intermediates into the pentose phosphate pathway that enhances the purine nucleotide synthesis [131,142]. NRF2 also promotes glutamine metabolism, thereby enhancing glutathione synthesis and lactate production, which is designated as glutaminolysis. Inhibition of the pentose phosphate pathway in NRF2-active cells reduces their cell growth, and conversely, selective activation of the pentose phosphate pathway in the NRF2-inactive cells accelerates their proliferation. This effectively indicates that

facilitation of the pentose phosphate pathway is one of the major mechanisms the NRF2-mediated acceleration of cell proliferation.

4) Mitochondrial function and lifespan

A line of evidence has suggested a functional association between the KEAP1-NRF2 system and mitochondrial biogenesis and functions [143,144]. The NRF2-mediated regulation of mitochondrial functions is through the control of its substrate availability [143], and also NRF2 target gene products have been shown to participate in healthy mitochondrial maintenance, removing damaged mitochondria and inducing mitochondrial biogenesis [144]. Conversely, mitochondria themselves have been suggested to regulate NRF2 activity. A pool of SKN-1, which is a *C. elegans* of NRF2, is localized on the mitochondrial outer membrane, and the SKN-1 liberation from the mitochondria elicits the starvation response [145]. Precise understanding of interrelation between NRF2 and mitochondria may give a new insight of the NRF2-mediated regulation in cell proliferation and differentiation.

It should be noted that SKN-1 mediates diet-restriction-induced longevity [146-148], which has recently been shown accomplished by upregulation of collagens [149]. In analogy to the SKN-1 functions, NRF2 activity is positively correlated with longevity in rodents [150]. Therefore, NRF2 appears to govern lifespan extension, and an adequate level of NRF2 activation would contribute to the achievement of healthy aging.

Conclusions

As accumulating evidence has clarified the novel actions of NRF2 beyond the

cytoprotection against oxidative stress and xenobiotics, increasing attention has been paid to the impact of the KEAP1-NRF2 system on basic physiological processes, such as cell cycle regulation and energy metabolism. Though the transcriptome analysis has been a powerful approach to clarify the canonical function of NRF2 in cytoprotection, comprehensive information on the transcripts may not be sufficient to decipher the mechanistic aspects of the non-canonical NRF2 functions related to cell proliferation and differentiation. This is because the integrated outputs from the “NRF2 cistrome” are translated into protein modifications or alterations of metabolic flux distribution. Therefore, in addition to the transcriptome analysis, proteomic and metabolomic analyses are promising strategies for deciphering unsolved mechanisms of the non-canonical NRF2 functions.

One of the interesting questions is how NRF2 contributes to stem cell activity, particularly to the maintenance of stem cells. The excessive proliferation of stem cells for self-renewal often leads to aging and exhaustion of stem cells [56,57], while a substantial body of evidence describes that NRF2 activates the proliferation of tissue stem cells [70,71,74,75]. Whether the sustained activation of Nrf2 results in the depletion of stem cells or enhances self-renewal without exhaustion needs to be investigated to evaluate the clinical benefits of NRF2 activators in regenerative medicine. Furthermore, the elucidation of the molecular mechanisms underlying the differential roles of NRF2 in the pluripotent stem cells and tissue stem cells represents an interesting challenge.

Another remaining question in the NRF2 functions that have been reviewed in this article is what physiological stimuli employ NRF2 to regulate cell fate determination. A fascinating example is commensal bacteria residing in the alimentary

and respiratory tracts. Recently, bacterial flora has been shown to have a great impact on host health by modulating the activity of the immune system and systemic metabolism [151,152]. Miscellaneous signals or stresses generated from the microbiota may activate NRF2 for modulating proliferation and differentiation processes. How NRF2 activity is influenced by the microbiota and how NRF2 modulates the host cell behavior in response to the microbiota are attractive issues. Answering these questions would add a new layer of complexity to the NRF2-mediated control of cell proliferation and differentiation.

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Figure Legends

Figure 1. Downstream effectors of the NRF2-dependent regulation in cell proliferation and differentiation.

A. Elevation of canonical target genes of NRF2. NRF2 modifies cell activity by controlling intracellular levels of oxidative stress, which comprises an important regulatory axis of osteoclast differentiation (i) and maintenance of *Drosophila* ISCs (ii).

B. Novel NRF2 target genes that regulate cell proliferation and differentiation. NRF2-mediated induction of PPAR γ and CEBP β promotes adipocyte differentiation (i), whereas NRF2-mediated induction of RXR α inhibits adipocyte differentiation (ii). Enhanced NOTCH signaling due to NRF2 activation impacts on the maintenance and proliferation of stem cells and liver regeneration (iii). CXCR4 is indispensable for the maintenance of HSCs, and MyoD is a major regulator of SCs during skeletal muscle regeneration. In NRF2 deficiency, decreased expression levels of each factor leads to impairment of stem cell activity (iv).

ABSC, airway basal stem cell; HSC, hematopoietic stem cell; ISC, intestinal stem cell; MSC, mesenchymal stem cell; SC, satellite cell.

Table 1. NRF2 impacts on various cell type

Physiology	Cell Type
Bone Homeostasis	Osteoclast
	Osteoblast
	Osteoclast/Osteoblast
	Osteoclast/Osteoblast
Adipogenesis	Adipocyte
	White Adipose Tissue (WATs)
	White Adipose Tissue (WATs)
	Adipocyte
Stem Cell Activity	Intestinal Stem Cells (ISCs)
	Airway Basal Stem Cells (ABSCs)
	Embryonic Stem Cells (ESCs)
	Satellite Cells (SCs)
	Neuronal Progenitor Cells (NPCs)
	Hematopoietic Stem Cells (HSCs)
	HSCs
	HSCs
Liver Regeneration	Glioma Stem Cells (GSCs)
	Hepatocytes
	Liver
	Hepatocytes
	Liver
Energy Metaolism	Cancer Cells and MEFs
	Cancer Cells
Lifespan	<i>C. elegans</i>
	<i>C. elegans</i>

A Regulation of intracellular oxidative stress

(i) Inhibition of osteoclastogenesis

(ii) *Drosophila* ISC maintenance

B Induction of cell fate regulators

(i) PPAR γ , CEBP β ; Promotion of adipogenesis

(ii) RXR α ; Inhibition of adipogenesis

(iii) NOTCH; Stem cell regulation and liver regeneration

(iv) CXCR4, MyoD; Stem cell regulation