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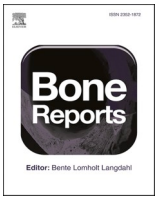

The role of the Nrf2/Keap1 signaling cascade in mechanobiology and bone health

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ABSTRACT

In conjunction with advancements in modern medicine, bone health is becoming an increasingly prevalent concern among a global population with an ever-growing life expectancy. Countless factors contribute to declining bone strength, and age exacerbates nearly all of them. The detrimental effects of bone loss have a profound impact on quality of life. As such, there is a great need for full exploration of potential therapeutic targets that may provide antiaging benefits and increase the life and strength of bone tissues. The Keap1-Nrf2 pathway is a promising avenue of this research. The cytoprotective and antioxidant functions of this pathway have been shown to mitigate the deleterious effects of oxidative stress on bone tissues, but the exact cellular and molecular mechanisms by which this occurs are not yet fully understood. Presently, refined animal and loading models are allowing exploration into the effect of the Keap1-Nrf2 pathway in a tissue-specific or even cell-specific manner. In addition, Nrf2 activators currently undergoing clinical trials can be utilized to investigate the particular cellular mechanisms at work in this cytoprotective cascade. Although the timing and dosing of treatment with Nrf2 activators need to be further investigated, these activators have great potential to be used clinically to prevent and treat osteoporosis.

1. Introduction

Bone health is a cardinal facet of wellness. Decreased bone quality can dramatically impact the quality of life of those suffering from bone disorders. These disorders vary in cause, severity, and age of onset; with the most common pathosis being fracture ((US), D. o. H. a. H. S., 2004). Other disease conditions include dislocations, degenerative processes, and cancer of the bone or surrounding tissues. The most common bone disease is osteoporosis (OP), with over 70 million people considered at-risk (Boyle et al., 2003). This disease presents as a gradual decline in bone mineral density (BMD) and is clinically defined by the World Health Organization as a BMD which lies 2.5 standard deviations or more below the average BMD of a young, healthy patient (Kanis et al., 2019). In addition to the debilitating physical and mental impacts of this disease, the economic burden is also substantial. In the US alone, costs associated with OP-related fractures reached nearly $17 billion in 2005, and with advances in medical care ever-increasing the average life expectancy, it has been estimated that the cost of these fractures will exceed $25 billion by the year 2025 (Burge et al., 2007).

This review aims to address the current state of research on the signaling cascade activated by master transcription factor Nrf2, and the prospective future research goals and therapeutic targets that could provide even more valuable insights into this powerful cytoprotective signaling cascade.

2. Pathogenesis of osteoporosis

Onset of osteoporosis (OP) can be described as either primary (originating with aging apart from another related disease) or secondary (resulting, at least in part, to a drug interaction or predisposing disease state). Primary OP occurs most commonly in post-menopausal women, as sex-steroid deficiency is a major contributing factor. Another leading cause of primary OP is oxidative stress, which is known to accumulate with advanced age (Hendrickx et al., 2015). While these OP risk factors associated with aging are naturally occurring and largely unavoidable, there is a complex network of compounding factors which also contribute to an individual's risk of developing OP. These include age-related genetic factors including predisposition to apoptosis and

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macroautophagy, as well as lifestyle influences like activity level, calcium and vitamin D intake, and risk behaviors (such as smoking and drinking) (Kanis et al., 2019; Hendrickx et al., 2015). Many of these predisposing conditions can occur in young and otherwise healthy individuals. Bone loss can afflict otherwise healthy patients due to immobilization after certain serious injuries or paralysis. Microgravity experienced in space flight is also known to compromise bone quality (Gerbaix et al., 2017; Orwoll et al., 2013; Vico et al., 2000). The most common route of secondary OP development is drug-induced OP, which is especially common following prolonged glucocorticoid treatment.

Treatment for OP is limited, especially since this condition frequently goes undiagnosed until after a fracture occurs (McCloskey et al., 2021). Treatment regimen often include vitamin D and calcium supplements, as well as lifestyle changes (e.g. exercise, increased activity, and fall prevention measures). Currently, the most commonly prescribed OP medications are antiresorptive drugs, including bisphosphonates (BPs) and denosumab (a RANKL inhibitor using human monoclonal antibodies). Less commonly, anabolic peptide hormones are used – often for patients with elevated fracture risk, or those intolerant to bisphosphonates (Kanis et al., 2019). These include teriparatide (a human parathyroid hormone derivative) and abaloparatide (a human parathyroid hormone-related protein derivative).

With all current treatment options, come adverse consequences. Potential side effects of long-term use include GI symptoms, musculoskeletal pain, transient hypocalcemia, ocular pain, increased risk of esophageal cancer, atrial fibrillation, osteonecrosis of the jaw and even increased risk of certain rare fractures (Shane et al., 2010; Lu et al., 2020; Tsvetov et al., 2020; Choi et al., 2017; Lewiecki, 2011). The link to cancer, however, remains scientifically uncertain and merits more research (Lu et al., 2020). Potential side effects of anabolic peptide hormone use include nausea, vomiting, headache, hypercalcemia, and increased risk of developing bone cancer (Tella et al., 2017; Minisola et al., 2019). Due to the combination of adverse side-effects, rigorous treatment regimes, cost of medications, and lack of perceived benefit; there is also a confounding problem with treatment nonadherence among patients (Kanis et al., 2019; Yeam et al., 2018). With the significant limitations and adverse side effects of currently-available treatments, there is a massive need for new therapeutic tools to treat OP. Nrf2 activators could provide bone support to at-risk individuals. These benefits, however, will need to be weighed against the potential for global adverse effects of long-term treatments with these compounds. One human trial of bardoxolone methyl as treatment for patients with Type 2 Diabetes and Stage 4 chronic kidney disease (CKD), published in 2013, was discontinued due to observed adverse effects including: weight loss, liver disorder, and even increased risk of death (although the etiology was not determined with certainty to be due to bardoxolone methyl use) (Chertow et al., 2014). Long-term dimethyl fumarate usage (a common treatment for multiple sclerosis and psoriasis) is also known to cause side effects including gastrointestinal upset, itching, and vision problems. As such, exploration into the targeted use of Nrf2 activators in bone treatment is an encouraging and necessary endeavor, which must be conducted with precision.

3. The Keap1-Nrf2 pathway

First characterized in 1994, nuclear factor erythroid 2-related factor 2 (Nrf2) is a member of the basic leucine zipper family of transcription factors (Moi et al., 1994). It was first discovered in a characterization study of K562 cells (an immortalized line derived from human myelogenous leukemia cell, which are known to be highly and reliably sensitive to NK lysis) (Moi et al., 1994). This study highlighted the homology between this newly discovered gene and NF-E2, its likelihood of unique properties attributed to its molecular structure, and a high degree of conservation between human and mouse genes (Moi et al., 1994). It heterodimerizes with small Maf transcription factors and activates the antioxidant response element (ARE) once translocated to the nucleus (Kensler et al., 2007). The ARE is the central cellular mechanism for alleviating oxidative stress. Since its discovery, the antioxidative therapeutic potential of Nrf2 has been a hot topic of research (Gold et al., 2012). It has earned the title of master regulator of cytoprotective and antioxidant genes, and has been referred to as the single most important classical signal pathway for cellular antioxidant function (Zhang et al., 2019a). Nrf2 also regulates mitochondrial biogenesis after stress. This has been demonstrated in skeletal tissue by Merry and Ristow in 2016 (Merry and Ristow, 2016). Mice treated with a combination (*R*)-α-lipoic acid and acetyl-L-carnatine (which activates Nrf2) have increased mitochondrial biogenesis in adipocytes (Shen et al., 2008). Nrf2-KO mice show decreased mitochondrial content in liver tissue (Dinkova-Kostova and Abramov, 2015). Recently, Nrf2 has even been suggested as a viable target for COVID-19 testing, as it has been shown that Nrf2 activation can ameliorate inflammatory respiratory symptoms as seen in pneumonia (Irmanida et al., 2020).

Under basal conditions, Nrf2 is lowly expressed – remaining in the cytoplasm bound to its sequestering protein, Kelch-like ECH-associated protein 1 (Keap1) (Kloska et al., 2019; Itoh et al., 1999). Keap1 prevents the translocation of Nrf2 to the nucleus and marks it for proteosomal degradation (Kensler et al., 2007; Itoh et al., 2010). Under stressed conditions, the cysteine residues which maintain the association with Nfr2 are oxidized, modifying their shape and releasing Nrf2 (Kensler et al., 2007; Kloska et al., 2019; Ichimura et al., 2013). Unbound Nrf2 is then free to translocate to the nucleus for activation, initiating the cytoprotective signaling cascade (see Fig. 1).

The antioxidant genes regulated by Nrf2 include thiol compounds (such as GSH) and non-thiol compounds (including polyphenols, vitamins, and enzymes) (Domazetovic et al., 2017). The list of cytoprotective genes regulated by Nrf2 is extensive, with many being essential for phase 2 detoxification (Itoh et al., 2010; Huang et al., 2015). Some known cytoprotective genes regulated by the Nrf2 signaling cascade are: heme oxygenase-1 (HO-1), NAD(*P*)H:quinone reductase (NQ01), glutathione S-transferase (GST), γ-glutamylcysteine synthetase (GCS), UDP-glucuronosyltransferases (UGT), and epoxide hydrolase (Alam et al., 1999; Thimmulappa et al., 2002). Other genes identified are involved in NADPH regeneration, metabolism of xenobiotics, and antioxidants (Thimmulappa et al., 2002). Gene expression biomarkers influenced by suppression or activation of Nrf2 in lung, liver and cancel cells have been reported (Cho et al., 2005; Namani et al., 2018; Rooney et al., 2020; Tonelli et al., 2018). Gene expression profile induced by down- or up-regulation of Nrf2 in bone cells remains to be investigated.

The powerful signaling cascade mediated by Nrf2 has been linked to many disease models, as well as conditions associated with the aging process (Chen and Maltagliati, 2017; Beyer et al., 2008). Nrf2 is expressed in all cell types, with basal expression being typically low due to Keap1 sequestering Nrf2 in the cytoplasm under basal conditions and marking it for degradation via ubiquitination. In certain cell types (specifically the immune cells in circulating blood) Nrf2 is expressed at a higher level, meaning more Nrf2 is translocated into the nucleus for translation and expression in those immunomodulatory cells (He et al., 2020). Nrf2 expression also increases under oxidative stress conditions. Being ubiquitously expressed, the therapeutic potential of the Nrf2 signaling cascade is immense (Kloska et al., 2019; Ichimura et al., 2013; Huang et al., 2015; Sun et al., 2015a; Bresciani et al., 2017). The decades of research into this pathway have yielded an abundance of novel insights into the molecular mechanisms which regulate the cytoprotective cascade; however, much of the investigation has focused on global knockout animal models. In these studies, Nrf2 expression is repressed constitutively via transgenic modifications, which applies to the whole animal. Such models can make it difficult for researchers to hone in on specific tissues or cellular mechanisms, as global effects of the whole body knockout can confound those observations. Only in recent years has research began to reveal the specific cellular mechanisms at work within the cascade (see Fig. 2 for a summary of factors linked to Nrf2 expression). The study of cell-specific models is essential to

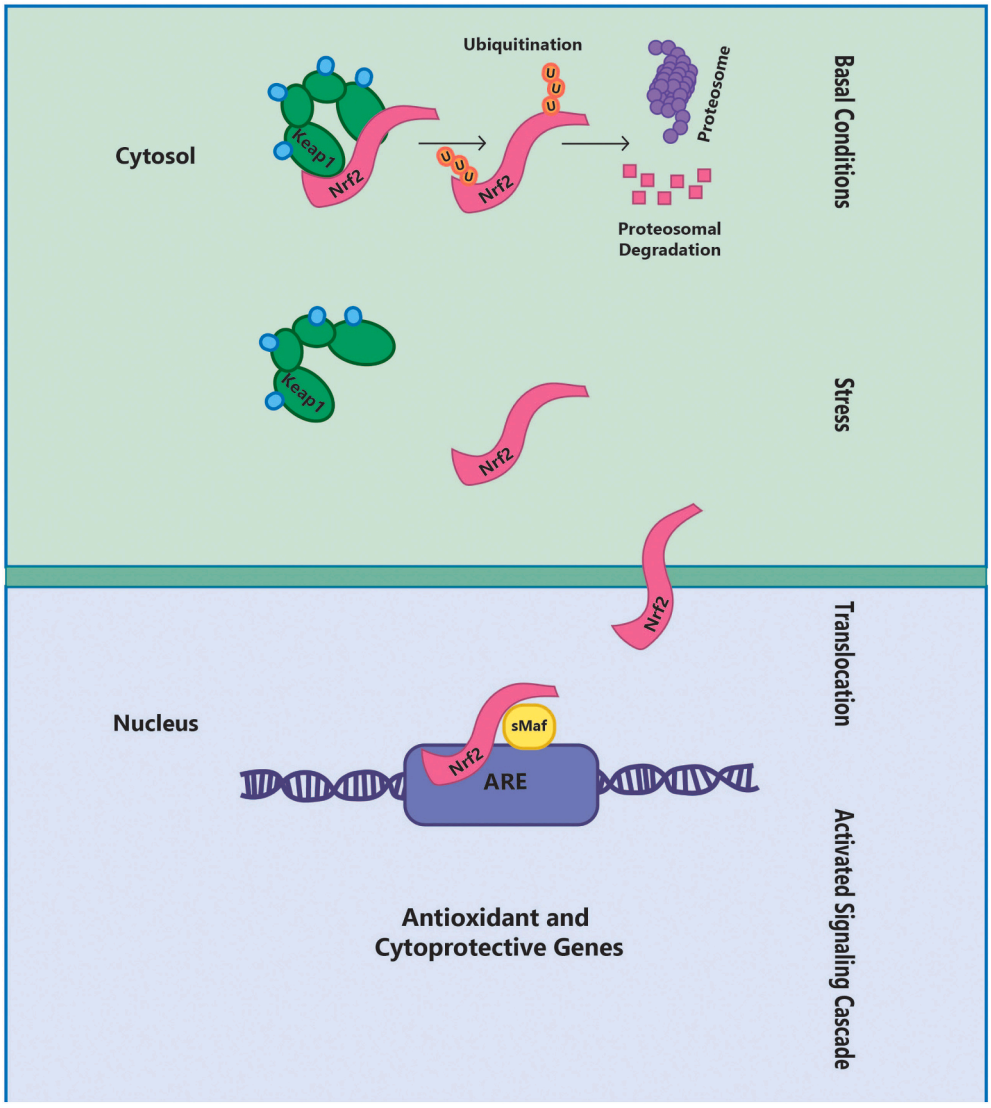

Fig. 1. Molecular mechanisms of the Keap1-Nrf2 pathway. Under basal conditions, Nrf2 is bound by its sequestering protein, Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. Keap1 prevents the translocation of Nrf2 to the nucleus and marks it for proteosomal degradation. Under stressed conditions, the cysteine residues which maintain the association with Nfr2 are oxidized, modifying their shape and releasing Nrf2. Unbound Nrf2 is then free to translocate to the nucleus for activation, initiating the cytoprotective signaling cascade.

understanding and treating bone disease. A common method of creating a cell- or tissue-specific knockout model is the Cre/Lox system, in which a Cre recombinase enzyme paired with specific targeted primers is bred into an animal line with constitutive expression of LoxP sites flanking the gene of interest (this is known as being 'floxed'). Floxed animals expressing the targeted Cre recombinase will therefore have a knockdown of the floxed gene only in cell population targeted by the cell-specific promoter which drives the Cre gene, allowing the remainder of the body to express normal levels of the gene and thereby minimizing global effects of gene knockdown. These models allow specific cell populations to be studied without the potentially compromising effects of global genetic modifications on the animals. Cell-specific models also provide valuable insights into which cell types are the most impactful in terms of therapeutic targets – allowing treatments and therapies to be as specific as possible, thus minimizing side effects and global impacts.

The detrimental effects of oxidative stress come into play when ROS levels are not adequately balanced by antioxidant function and become elevated (Domazetovic et al., 2017; Yao et al., 2020). Some behaviors and disease states are characterized by elevated and persistent oxidative stress, including heavy smoking, long-term alcohol abuse, type-2 diabetes mellitus (T2DM), osteoporosis, Alzheimer's and Huntington's disease. In many cases, although a disease state has been associated with oxidative stress, treatment via administration of antioxidants, such as coenzyme Q10, as the sole therapy has not been proven successful mainly due to under-exposure of drug concentration and timing of treatment at the target tissues (Bresciani et al., 2017). In these instances, there is potential that therapeutic intervention into the Keap1-Nrf2 pathway could provide a more effective treatment as a part of a compound or combined therapy which activates the innate cytoprotective cascade for a controlled duration of time (Bresciani et al., 2017).

4. Nrf2 activators

Several methods of Nrf2 regulation have been demonstrated successfully. Many natural and pharmacological inducers have been shown to activate Nrf2 (Ajiboye et al., 2014). One such activator is the compound bardoxolone methyl (CDDO-Me), an investigational drug produced to combat chronic disorders through activation of the Keap1/Nrf2 pathway (Reata, 2020). CDDO-Me is a synthetic triterpenoid derivative which interacts in a reversible manner with the cysteine residues of Keap1 (Zhou et al., 2014). CDDO-Me was well tolerated with a daily dose of 900 mg (Hong et al., 2012). In a Phase 2 clinical trial, CDDO-ME at an oral dose of 25, 75 and 150 mg once daily for 52 weeks has been shown to be associated with improvement renal function in patients with advanced chronic kidney diseases and type 2 diabetes (Pergola et al., 2011). Post-hoc analyses from CDDO-ME study shows CDDO-ME

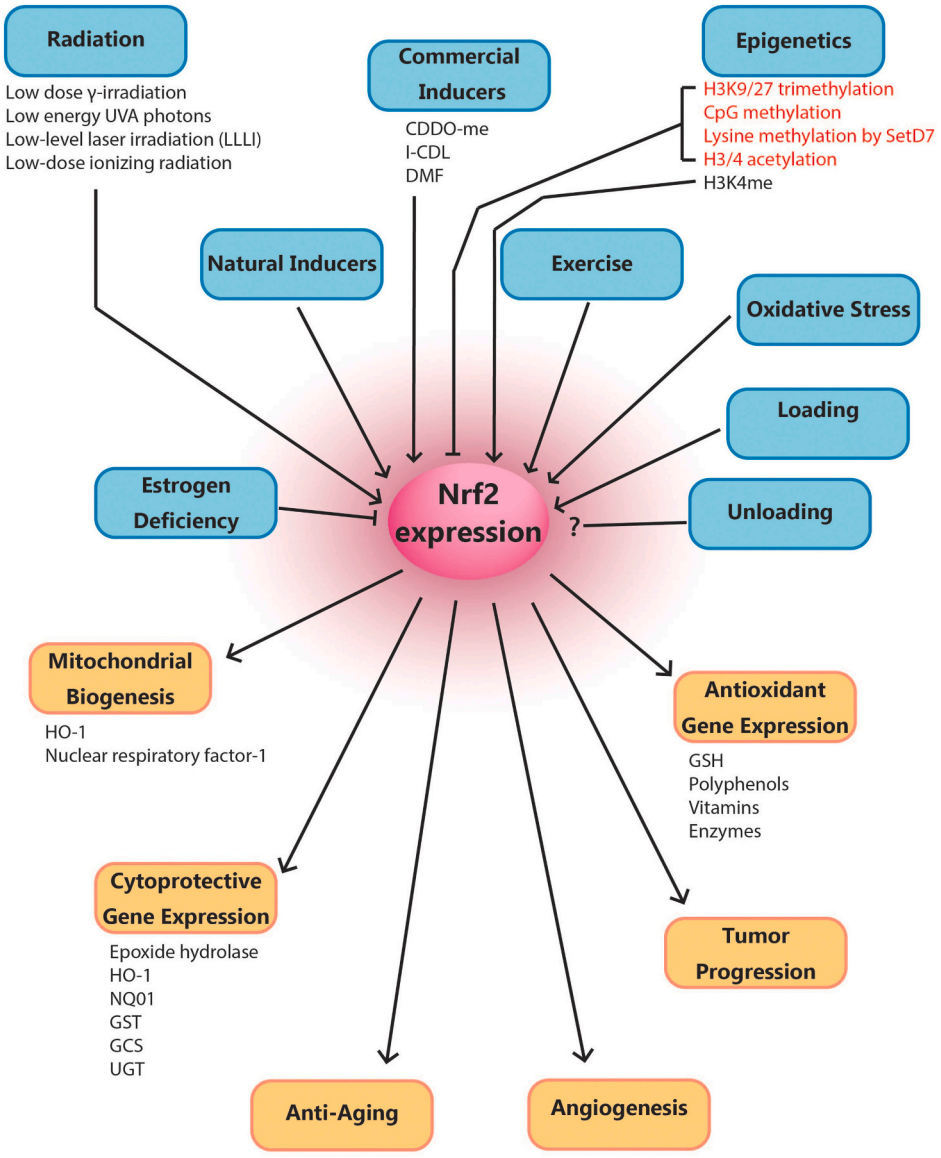

Fig. 2. Biological factors contributing to and resulting from Nrf2 expression. Pointed arrows indicate positive regulation. Barred arrows indicate inhibition. Factors which influence Nrf2 expression are displayed in blue, while downstream implications of Nrf2 expression are shown in orange. Lists below bubbles display specific compounds involved, with red font representing inhibition and black font indicating activation (Kensler et al., 2007; Merry and Ristow, 2016; Itoh et al., 2010; Domazetovic et al., 2017; Huang et al., 2015; Alam et al., 1999; Thimmulappa et al., 2002; Chen and Maltagliati, 2017; Ajiboye et al., 2014; Zhou et al., 2014; Yamaguchi et al., 2018; Liu et al., 2018; Florczyk et al., 2014; Toledo-Arruda et al., 2020; Zhou et al., 2020; Cheng et al., 2016; Tsukimoto et al., 2010; Liu et al., 2019b; Rysava et al., 2020; Wang et al., 2020b; McDonald et al., 2010; Bellanti et al., 2013; Zimta et al., 2019).

(20 mg, once daily) preserves kidney function and may delay the onset of end stage of renal disease in patients with type 2 diabetes and stage 4 chronic kidney disease (Chin et al., 2018). This compound has shown promising results in clinical trials as a potential therapy for many disease states including chronic kidney disease (CKD) and certain cancers, with relatively mild side effects as compared to existing treatments (Zhou et al., 2014). Bardoxolone Methyl has recently been filed to be approved as a treatment for CKD caused by Alport Syndrome (a disease with no currently approved treatments), and the action on that filing is expected by February of 2022 (Reata, 2020). Another Nrf2 inducer which has been approved by FDA, dimethyl fumarate (DMF), has been found to inhibit osteoclastogenesis (Yamaguchi et al., 2018). In Germany, this compound is part of an approved psoriasis treatment (trade name Fumaderm) (Mrowietz et al., 2007). In the UK, it is approved for treatment of multiple sclerosis (trade name Skilarence). It is also approved for use treating multiple sclerosis in the US (Biogen brand name Tecfidera) (Booth et al., 2016), and a generic version has been approved in 2020.

Some of the natural Nrf2 activators include flavonoids, phenols, terpenoids, alkaloids, sulforaphane, avenanthramides, and gastrodin (Tavakkoli et al., 2019; Liu et al., 2018). Upon recent investigation, several traditional Chinese medicinal agents have been found to activate Nrf2 as their method of action. These include Fufang Lurong Jiangu Capsule (FLJC) (Jin et al., 2020) and Naringin (Yu et al., 2021). Some of these activators have been shown to have such high bioavailability that they are even likely to effectively cross the blood-brain barrier, namely sulforophane and berberine (Tavakkoli et al., 2019). These compounds provide a variety of tools researchers can and are utilizing in the investigation of the potential for Nrf2 activation as a treatment for a myriad of disease conditions. Could this pathway also serve as an avenue to better bone health? Careful consideration of the dosages, side effects, and possible combination therapies will need further investigations in order to find out.

5. Functional Nrf2 signaling cascade and bone homeostasis

Nrf2 is poised as a promising target for potential prevention or treatment of bone diseases. Oxidative stress has been found to positively correlate with the loss of bone mass associated with OP development, and has been found to decrease bone turnover and impede fracture healing (Kubo et al., 2019). A study of male Wistar rats measured blood plasma levels of markers of oxidative stress and analyzed femur characteristics, and found that increased oxidative stress and bone loss were observed with aging (Zhang et al., 2011). As such, many studies have

targeted the Nrf2 antioxidant potential in bone tissues, and found evidence that antioxidants play an important role in the dynamics of bone remodeling, from the activation of bone cells to the gain and loss of overall bone mass (Domazetovic et al., 2017; Sun et al., 2015a; Pellegrini et al., 2017; Yin et al., 2020). Nrf2 has been suggested as a therapeutic target for the treatment of myeloma bone disease (Yen et al., 2020). It has also been identified as a potential contributing factor in the progression of Paget's Disease of Bone (PDB) (Wright et al., 2013). The Keap1-Nrf2 pathway has also been linked to regulation of murine bone metabolism in recent years. Nrf2 deficiency causes increased oxidative stress and increased bone turnover, resulting in diminished bone microarchitecture (Ibáñez et al., 2014). In adult male mice, Nrf2 deficiency has been shown to diminish load-driven bone formation as well as bone strength, when compared to littermate controls (Sun et al., 2015b). Another study, which investigated both sexes, found that moderate activation of Nrf2 via partial deletion of Keap1 led to a significant increase in bone formation rate in males, but not in females (Yin et al., 2020). A recent study found that Nrf2 plays a key role in osteocyte gene expression, and Nrf2 ablation of those osteocytes (through cre/lox tamoxifen-induced cKO of 3 week old mice) results in increased osteoclastogenesis and subsequent bone loss (Sánchez-de-Diego et al., 2021). Some contrasting results have also been obtained. A 2014 study using mRNA analysis on bone marrow cells flushed from tibiae of 7-week old Nrf2 KO mice found evidence that Nrf2 inhibited both osteoclastogenesis and osteoblastogenesis (Park et al., 2014). Also, reports from one research group suggest global Nrf2 hyperactivation via global Keap1 deletion led to bone hypoplasia due to decreased osteoclast differentiation (Yoshida et al., 2018). It is worth noting, however, that this contrasting study utilized a specialized mouse model. These mice had a deletion of esophageal Nrf2, which allowed a complete Keap1 knockout (a condition which is neonatally lethal in all other mouse lines), and the line was shown to have systemic effects of this deletion, such as diminished bone and body growth. Other sources of bone loss have also been linked to the Keap1-Nrf2 pathway. Nrf2 deficient mice experience greater bone loss following ionizing radiation exposure (Rana et al., 2012) and inorganic arsenic intake (Liu et al., 2019a).

Antioxidants play a significant role in overall health, by mediating a biological system's response to stress. Cellular stress originates from a multitude of sources. Some exogenous stressors include drugs, pollutants, and food additives. These foreign substances, known as xenobiotics, contribute to cellular redox reactions which form reactive oxygen species (ROS) (Pagano, 2002). Some ROS, however, are produced naturally – as byproducts of normal metabolic processes (Yao et al., 2020). Although these ROS are oxidants and cellular stressors, they play an essential role in some cellular signaling pathways, and are therefore necessary at low physiological levels (Yao et al., 2020). Complete elimination of ROS would greatly impair functions such as bone marrow homeostasis (Yen and Hsiao, 2018).

Another possible arena of significance for the Keap1-Nrf2 pathway in bone health involves the bone marrow, an indispensable therapeutic resource, home to hematopoiesis and the source of mesenchymal stem cells. In one study, bone marrow cells such as hematopoietic stem cells (HSCs) and proangiogenic cells (PACs), showed increased localized Nrf2 expression when treated with angiogenic cytokines; a study that was conducted both in vitro and in vivo in mice following hind limb ischemia – and suggests that Nrf2 may play an important role in mobilizing cells from the bone marrow to the site of injury to promote angeogenesis (Florczyk et al., 2014). A recent study using BMSCs isolated from the bone marrow of rabbits found evidence that preconditioning cells with oxidative stress in the form of pretreatment with H_2O_2 (preconditioning) increased the efficiency with which Nrf2 was translocated into the nucleus, leading to increased levels of antioxidant enzymes such as SOD, CAT, NQO1, and HO-1 (Zhang et al., 2019a). In a study of male rats with lipopolysaccharide(LPS)-induced sepsis, those who treated with bone marrow-derived mesenchymal stem cells (BMSCs) showed improved tissue regeneration and decreased apoptosis – traits attributed to the activation of the Nrf2 pathway in the BMSC-treated animals (Selim et al., 2019). The homing ability of collected stem cells was analyzed, and also suggested that the same BMSCs can go on to provide further Nrf2 activation (and subsequent protection against oxidative stress) elsewhere in the body (Selim et al., 2019). These discoveries suggest that therapeutic strategies may be able to enhance the manner by which cell or tissue types utilize the Nrf2 signaling pathway when necessary.

6. Nrf2 and exercise

Physical inactivity has been shown to significantly increase the likelihood of death (Bouchard and Shephard, 1994). All safe forms of exercise and physical activity are therefore potential sources of extended livelihood (Blair et al., 2001). Studies of skeletal muscle have linked some of the healthful adaptative responses to exercise specifically to Nrf2 activation (Merry and Ristow, 2016). This concept has yet to be fully explored in the context of bone tissues. When it comes to increasing and maintaining BMD specifically, load-bearing resistance exercise has shown to be the most impactful form of exercise (Warburton et al., 2006).

The mouse hindlimb unloading (HU) model achieved through tail suspension was developed in an effort to mimic spaceflight conditions and astronaut bone loss (Wronski and Morey-Holton, 1987). The HU model animal also provides an opportunity to study disuse associated with bedrest or sedentary lifestyles that are frequently associated with decreased bone health (Tahimic et al., 2019).

Skeletal muscle is intimately intertwined with development of osteoporosis. A recent study in rats suggests that Nrf2 activation reduced incidence of osteoporosis, and linked this effect in part to the skeletal muscle-secreted novel actin Irisin and its downstream regulation of the NLR family pyrin domain containing protein 3 inflammasome which exhibited a protective effect against osteoblast apoptosis (Xu et al., 2020). Skeletal muscle Nrf2 is activated in response to acute exercise, leading to mitochondrial biogenesis and increased antioxidant function (Merry and Ristow, 2016). Skeletal muscle is known to produce ROS (Powers and Jackson, 2008), which could lead to Nrf2 activation in surrounding tissues, and may impact the bone microenvironment with which is it intimately associated. As such, more research is necessary in both the field of bone mechanobiology and skeletal muscle biology in order to gain a better understanding of the precise mechanisms by which exercise impacts muscle and bone health.

7. Mitigation of bone loss via Nrf2 regulation

One of the most prevalent and impactful bone diseases is osteoporosis. Osteoporosis is characterized by bone loss, which leads bones to become brittle, fragile, and susceptible to fractures. Many of life's most common circumstances can increase an individual's risk of bone loss. This includes the process of aging, which is often accompanied by estrogen deficiency and decreased load bearing activity – both of which are correlated with bone loss and increased fracture risk. Ovariectomized (OVX) adult female mice can provide a post-menopausal experimental animal model. After the ovariectomy, these mice take on characteristics analogous to post-menopausal osteoporosis. Estrogen deficiency may lead to an elevated level of ROS. However, it remains to be investigated if Nrf2 activation can prevent or restore bone loss caused by loss of estrogen in vivo.

There are iatrogenic sources of bone loss as well, such as radiation, glucocorticoid treatment, and comorbidities of diabetes. While endogenous levels of glucocorticoids correlate with osteoblastogenesis and anabolism of bone tissue, exogenous glucocorticoid treatment has been found to have a deleterious effect on osteoblast cells (Hartmann et al., 2016). Natural Nrf2 activator gastrodin was used in a study to attenuate the osteoblast dysfunction induced by high exposure to glucocorticoid dexamethasone via osteogenic effects of the Nrf2 pathway (Liu et al., 2018). Patients receiving long term GC treatment often express elevated

levels of phosphatase and tensin homolog (PTEN); a tumor suppressor gene that promotes cell apoptosis. A recent rat study investigating the effects of VO-OHpic, (a powerful inhibitor of PTEN) activates the Nrf2 pathway, and this activation is critical to the protective effect of VO-OHpic. This indicates that PTEN may repress Nrf2 activity (Yao et al., 2020), suggesting that activation of Nrf2 may help to prevent bone loss caused by long term use of GC.

8. Cellular and molecular mechanisms involved in Nrf2 regulation

Are all bone cells equally impacted by the Nrf2 pathway? The cellular mechanism is not fully understood at this time, but studies have found evidence that all of the major bone cells are surely affected by oxidative stress. Studies have linked elevated ROS levels to a reduction in osteoblastogenesis (Mody et al., 2001). Another linked elevated ROS levels to increased osteoclastogenesis, finding decreased bone mineral density at several measured points exposed to oxidative stress (Baek et al., 2010). Nrf2 has been shown to regulate transcription of MYC, a factor necessary for osteoclastogenesis (Park et al., 2020). Elevated ROS could lead to apoptosis of osteocytes, leading to an imbalance in bone turnover which favors osteoclastogenesis and leads to bone loss (Domazetovic et al., 2017) While the cellular mechanisms have not been fully elucidated at this time, it is clear that oxidative stress has a distinct and significant impact on each of the cell types responsible for maintaining bone health.

9. Sex- and age-dependent differences in Nrf2 expression and antioxidant function

Nrf2 expression and activity decrease with aging. A previous study has shown aged rat liver displays a significant loss of total and nuclear Nrf2 content and its transcriptional activity, which significantly reduce the glutathione synthesis (Suh et al., 2004). Glutathione is a key pathway in restoration of redox homeostasis to mitigate oxidative stress. Nrf2 expression and activity in aged bone cells have not elucidated and need to be investigated in the future.

In addition to the sex-specific differences previously described here, one study found that Nrf2 deletion decreased bone formation rate in females while increasing bone formation rate in males, suggesting that Nrf2 in the bone microenvironment may be required by females, yet unnecessary or detrimental to males (Pellegrini et al., 2017). The same study evaluated aged test groups of mice, and found that aged Nrf2 KO mice display sex-specific differences in antioxidant function, with females expressing lower levels of detoxifying and antioxidant enzymes while males showed no difference in enzyme expression when compared to littermate controls (Pellegrini et al., 2017). As these results were not mirrored in the young test groups, it is evident that Nrf2 affects mice differently depending upon both age and sex.

Age may also be a significant contributing factor to the role and extent of Nrf2 activation on biological systems. Oxidative stress contributes directly to aging and age-related diseases, so antioxidant factors such as Nrf2 are likely a great source of potential antiaging benefits (Kensler et al., 2007; Callaway and Jiang, 2015). Several studies have implicated Nrf2 as a source of antiaging benefits for various tissue types, including: liver (Wang et al., 2020a; Farouk Kamel et al., 2018), fibroblasts (Hseu et al., 2020), salivary glands (Sisca Meida et al., 2020) and skin (Zhong et al., 2019). Nrf2 has also been linked to increased longevity in whole animal models including mouse (Travis et al., 2011), *Caenorhabditis elegans* (Yan et al., 2017), and *Drosophila melanogaster* (Zhang et al., 2019b). Interestingly, anti-aging effects of Nrf2 activation may depend on sex. One study shows that Nrf2 activation extended median lifespan in male mice only (Strong et al., 2016). Another study demonstrated positive sex-dependent effects of Nrf2 activation in senescent mice: prevention of the age-related decrease in body weight only in old females and improvement of motor function only in males (Berry et al., 2020). Therefore, the role of Nrf2 signaling in bone cells needs to be carefully studied in both male and female aged animals.

10. Can this powerful pathway be controlled in a targeted manner?

While Nrf2 activation is widely hailed for its potential in cytoprotection and anti-aging, it is worth noting that the ideal balance of expression should be exhaustively studied. It is entirely possible that Nrf2 activation could lead to unintended consequences. There have been some conflicting reports which presented evidence both for and against the effects of Nrf2 on angiogenesis. A recent study found that Nrf2 supported angiogenesis in cases of osteonecrosis of the femoral head and endothelial progenitor cells (Yao et al., 2020). The role of Nrf2 expression in cancer development is also unclear. It has been suggested that the cytoprotective action of activated Nrf2 can contribute to the successful proliferation of aggressive malignant cancers (Mitsuishi et al., 2012). A recent study of human breast cancer patients, however, reported that higher Nrf2 expression correlated with higher survivability, greater drug sensitivity, and more thorough tumor infiltration by immune cells (Oshi et al., 2021). These are important concerns to keep in mind when experimentally inducing Nrf2 overexpression, as its inherent protective capabilities may inadvertently protect detrimental biological systems in this way. In the future, it will be paramount to understand the mechanisms by which this pathway affects different bone cells if a safe and effective therapeutic protocol is to be developed.

11. Current and future research

The promising and far-reaching effects of the Keap1-Nrf2 pathway extend to nearly every tissue and body system, and much evidence has been recently brought forward to suggest that Nrf2 may be a valuable therapeutic target for the prevention and treatment of bone diseases, and maintenance of bone health with aging. Advantageously, Nrf2 activators have also been suggested as treatment for other disorders – especially chronic metabolic conditions and those associated with aging (Irmanida et al., 2020; Hseu et al., 2020; Zhong et al., 2019; Chen et al., 2019). The potential future for Nrf2 as a general target for health and antiaging is highly encouraging. Future research should endeavor to home in on the specific cell-level mechanisms by which this profound cytoprotective cascade functions. In bone, which has been shown to respond to Nrf2 activation and deletion, further study should investigate precisely which cells are responsible for the tissue-level changes. In addition, studies should attempt to analyze the microenvironmental signals associated with activation and deletion of Nrf2. Proteomics and RNA sequencing should be capitalized on in the investigation of this pathway. A research group recently published a protocol by which nuclear accumulation can be measured using targeted mass spectrometry (Östreicher et al., 2019). This approach could be a valuable tool for quantifying Nrf2 upregulation at a single-cell level.

Care should be taken to identify instances where manipulation of Nrf2 expression levels (such as global or partial Keap1 deletion) may have systemic effects on the animal intrinsic to the change in Keap1 – and not necessarily a direct result of the change in Nrf2. Also, all possible efforts should be made to conduct future research using both male and female test groups, as well as appropriately aged animals, as it is evident that the Keap1-Nrf2 pathway varies in impact depending on sex and the same can be said of age (Pellegrini et al., 2017). While the skeleton develops to its maximum potential in the young, the incidence of bone disease increases drastically with age. Since studies have already illustrated that aged test groups respond differently to young adult test groups, the age of the animals should be considered based on the conditions being studied.

In addition, other mechanisms of amplifying antioxidant functions should be explored in relation to bone growth as well. One prospective line is the mitoCAT-FS line of mice, which inducibly express human

catalase enzyme when the stop codon is removed via cre/lox breeding system (Ucer et al., 2017). Catalase deactivates ROS (Kensler et al., 2007). These mice exhibit prolonged life spans with significant reductions in age-related pathologies including cataract development, cardiac complications, and oxidative stress (Samuel et al., 2005; Dai et al., 2011; Treuting et al., 2008).

12. Conclusion

In conclusion, the mounting evidence has made it clear that Nrf2 is a promising target of research. It lends itself to global activation studies but is also a promising target for development of precisely targeted therapeutic tactics. It is likely to have a significant role in all tissues and body systems, and its cytoprotective and anti-aging properties have seemingly endless potential. There is still much to uncover about this pathway. Until the prevalent unknown variables of Nrf2 activation are explored in depth, the potential of Nrf2 as a therapeutic target for bone health are unclear. Development of representative animal models with minimized systemic effects of genetic modifications, as well as cell-level and molecular-level studies will surely expand the knowledge base on this topic, and may empower the scientific community with the ability to utilize the Nrf2 pathway to the benefit of human health.

Declaration of competing interest

None.

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Glossary

ARE: antioxidant response element
BMSC: bone marrow-derived mesenchymal stem cell
CDDO-Me: dimethyl fumarate, an Nrf2 activator
cKO: conditional knockout
CKD: chronic kidney disease
DMF: dimethyl fumarate, an Nrf2 inducer
EC_{50}: half maximal effective concentration
GCS: γ-glutamylcysteine synthetase
GST: glutathione S-transferase
HO-1: heme oxygenase-1
Keap1: Kelch-like ECH-associated protein 1, a homodimeric protein which sequesters and regulates degradation of Nrf2
LPS: lipopolysaccharide
MS: multiple sclerosis
NQ01: NAD(*P*)H:quinone reductase
Nrf2: nuclear factor erythroid 2-related factor 2, a basic leucine zipper transcription factor
OP: osteoporosis
ROS: reactive oxygen species
UGT: UDP-glucuronosyltransferases