The role of nuclear factor-E2-related factor 1 in oxidative stress response in osteoblast cells

So Young Park

Department of Medicine

The Graduate School, Yonsei University

The role of nuclear factor-E2-related factor 1 in oxidative stress response in osteoblast cells

So Young Park

Department of Medicine

The Graduate School, Yonsei University

The role of nuclear factor-E2-related factor 1 in oxidative stress response in osteoblast cells

Directed by Professor Sung-Kil Lim

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

So Young Park

June 2010

This certifies that the Doctoral Dissertation
of So Young Park is approved.

Thesis Supervisor : Sung-Kil Lim

Thesis Committee Member#1 : Myoung Hee Kim

Thesis Committee Member#2 : Seong-Hwan Moon

Thesis Committee Member#3 : Han-Sung Jung

Thesis Committee Member#4 : Man-Wook Hur

The Graduate School
Yonsei University

June 2010

ACKNOWLEDGEMENTS

I would like to begin by expressing my sincerest appreciation to my supervisor Professor Sung-Kil Lim for his tremendous guidance, encouragement, support and everything else connected to this work. I would also like to express my gratitude to the committee members Professor Myoung Hee Kim, Professor Seong-Hwan Moon, Professor Han-Sung Jung, and Professor Man-Wook Hur for their enthusiasm, critical review of the manuscript and their valuable comments and advices.

I am grateful to the faculty and colleagues of the department of endocrinology, Cheil general hospital for their supports.

Last but not least, I would like to thank my family members who have constant confidence in me for all these years.

So Young Park

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The role of nuclear factor-E2-related factor 1 in oxidative stress response in osteoblast cells

So Young Park

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Sung-Kil Lim)

Oxidative stress and antioxidants have been shown to regulate cellular function and metabolism. A normal balance between oxidants and antioxidants is needed for the maintenance of equilibrium between osteoblast-osteoclast. Excessive oxidants induce many defense mechanisms such as antioxidant enzymes. The antioxidant response element (ARE) is found in the promoters of antioxidant genes and has been reported to bind diverse transcriptional factors.

Nuclear factor-E2-related factor 1 (Nrf1, NFE2L1), a member of the Cap'n'Collar-basic leucine zipper protein family, is known to bind the ARE and regulate the expression of a number of genes involved in oxidative stress, cellular differentiation and inflammation.

The purpose of this study was to examine the effects of Nrf1 on the response to oxidative stress in osteoblastic MC3T3E1 cells. This study also examined the effects of Nrf1 on osteoblastic proliferation and

differentiation under unstimulated and oxidative stress conditions.

The expression of Nrf1 mRNA was upregulated with lipopolysaccharide (LPS) treatment in MC3T3E1 cells. LPS induced formation of intracellular reactive oxygen species (ROS) in MC3T3E1 cells. Transfection with siRNA against Nrf1 suppressed the expression of antioxidant genes including metallothionein 2 (MT2), glutamate-cysteine ligase catalytic subunit (GCLC), and glutathione peroxidase 1 (GPx1) in LPS-treated MC3T3E1 cells. Knockdown of Nrf1 expression was not sensitized to MC3T3E1 cells proliferation under unstimulated or oxidative stress conditions. In contrast to previous studies, knockdown of Nrf1 expression did not affect osteoblast differentiation in MC3T3E1 cells. However, knockdown of Nrf1 expression showed increased cartilage nodules in embryonal mesenchymal cells.

In conclusion, Nrf1 contributes to the expression of several antioxidant genes such as MT2, GCLC, and GPx1 in osteoblasts under oxidative stress. This *in vitro* study suggests that Nrf1 alone does not have a decisive effect on regulating osteoblast proliferation or differentiation. However, Nrf1 might have a variable effect on cellular differentiation according to the stage of development. Further study is needed to evaluate the comprehensive mechanisms of the Nrf1-antioxidant pathway in various pathologic conditions.

Key words: nuclear factor-E2-related factor 1, Nrf1, oxidative stress, osteoblast

The role of nuclear factor-E2-related factor 1 in oxidative stress response in osteoblast cells

So Young Park

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Sung-Kil Lim)

I. INTRODUCTION

Oxidative stress resulting from abnormally excessive levels of reactive oxygen species (ROS) and low levels of antioxidants is implicated in causing oxidative damage to the extracellular matrix and deterioration of cell metabolism or viability.[1] The delicate balance between oxidants and antioxidants is achieved by redox regulation to protect living organisms from various oxidative stresses. Oxidants/antioxidants reaction is partly related to bone metabolism to maintain equilibrium between osteoblast-osteoclast.[2]

Living organisms have developed several defense mechanisms against oxidative stress, including preventative mechanisms, repair mechanisms, physical defenses and antioxidant defenses.[3] Two types of antioxidant enzymes involved in the regulation of cell antioxidant metabolism and the detoxification of xenobiotics are regulated by antioxidant response element (ARE). The ARE is found in the promoters of antioxidant genes and is activated by phenols, heavy metal atoms, hydrogen peroxide (H_2O_2), and inflammatory stimuli, etc.[4] The ARE has been reported to bind diverse transcriptional factors.

Lipopolysaccharide (LPS) is a constituent of the cell wall outer membrane of gram-negative bacteria and has variable biological effects such as immune and inflammatory responses.[5] LPS increases production of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and prostaglandin E2 (PGE2). TNF-α and IL-1 can generate ROS in nonphagocytic cells.[3] LPS stimulates osteoblasts to secret osteolytic factors, including IL-1, IL-6, and PGE2.[6] LPS is also involved in the suppression of bone sialoprotein (BSP), a mineralized tissue-specific protein in osteoblast-like ROS 17/2.8 cells.[7]

Nuclear factor-E2-related factor 1 (Nrf1, NFE2L1) is a basic leucine zipper protein (bZIP) in the Cap-N-Collar (CNC) transcriptional factor family and controls ARE-driven genes.[8] The CNC-bZIP subfamily has highly conserved DNA-binding and protein-dimerization domains in basic structure, and comprises p45-NF-E2, Nrf1, Nrf2 (NFE2L2), Nrf3, Bach1, and Bach2.[8-13] The CNC-bZIP factors function as obligate heterodimers with small Maf proteins (Maf G, Maf K, Maf F) for DNA binding.[14]

Nrf1 was originally suggested to have a role in beta-globin gene expression in erythroid cells, but Nrf1 has been shown to bind the ARE and regulate the expression of many genes involved in oxidative stress, cellular differentiation, and inflammation.[15]

Nrf1 can protect cells from oxidative stress by regulating genes encoding enzymes concerned in glutathione (GSH) biosynthesis and other oxidative defense enzymes. Fibroblasts from Nrf1-deficient embryos showed reduced expression of glutamate-cysteine ligase modifier subunit (GCLM) and glutathione synthetase (GSS).[16,17] Overexpression of Nrf1 in cells up-regulated activities of glutamate-cysteine ligase catalytic subunit (GCLC) and GSS.[18] Furthermore, Nrf1-deficient (Nrf1-/-) hepatocytes down-regulated metallothionein (MT)-1 and -2.[19]

Nrf1 is expressed in skeletal cells and regulates genes related to cellular differentiation. Nrf1 was reported to interact with cytidine-cytidine-adenosine

-adenosine-thymidine (CCAAT) enhancer binding protein (C/EBP)β and function as a negative regulator during odontoblast differentiation.[20] Ascorbic acid treatment induced osterix expression in bone marrow stromal cells (BMS) derived from gulonolactone oxidase-deficient mice, which is mediated by Nrf1 binding ARE of osterix gene.[21] Osteoblast-specific Nrf1 knockout mice showed partial impairment of osteoblast differentiation and bone formation.[22]

The aim of this study was to evaluate the role of Nrf1 in osteoblast response to oxidative stress. The present study also investigated whether Nrf1 has an influence on osteoblastic proliferation and differentiation in MC3T3E1 cells under unstimulated and oxidative stress conditions.

II. MATERIALS AND METHODS

1. Cell culture

The murine calvaria-derived MC3T3E1 osteoblast-like cell line was used for the experiments. MC3T3E1 cells were maintained in α-modified minimum essential medium (α-MEM) containing antibiotics and 10% fetal bovine serum. This basic medium was replenished every 3 days. For the osteoblastic differentiation, cultures were transferred to α-MEM supplemented with 50 μg/ml L-ascorbic acid and 10 mM β-glycerophosphate.

2. Induction of oxidative stress

MC3T3E1 cells were seeded in 96-well plates or 6-well plates. Cells were then treated with oxidative stress-inducing agents. H_2O_2 (Sigma-Aldrich, Saint Louis, MO) was used at a concentration range of 0 ~ 400 μM. LPS (Sigma-Aldrich, Saint Louis, MO) was used at a concentration range of 0 ~ 10 μg/ml.

3. Transfection of small interfering RNA (siRNA)

MC3T3E1 cells were plated in 96-well plates or 6-well plates. After overnight culture, the cells were transfected using Lipofectamine PLUS reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Each transfection assay was performed with control siRNA or Nrf1 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA).

4. RNA isolation and quantitative real-time PCR (RT-PCR)

Cultured cells were superficially washed with phosphate-buffered saline (PBS), followed by the extraction of total RNA using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's standard instructions. Samples (2 μg) of total RNA were reverse transcribed, followed by oligo (dT) primer and

MMLV Reverse Transcriptase (final volume; 25 μl). Aliquots of 2 μl cDNA were used as templates for real-time PCR. PCR amplification was performed with 2x SYBR® Premix Ex Taq™ (Takara Bio Inc., Shiga, Japan) and 10 pmol forward and reverse primers using Thermal Cycler DICE Real Time System (Takara Bio Inc., Shiga, Japan). Reactions were performed for 45 cycles of 95 ℃ for 10 s, 60 ℃ for 15 s, and 72 ℃ for 30 s. Primers are listed in Table 1.

Table 1. Primers used

Genes	Forward (5'-3')	Reverse (5'-3')
β-actin	CCGCGAGCACAGCTTCTT	CCCACGATGGAGGGGAATAC
Nrf1	GGAGAGCTTCCCTGCACAGT	TTACTTCCATAGCCTGCATTTCC
ALP	AATTCTGCCTCCTTCCACCA	CGGAACTCCTGACCCTTGAC
MT1	ATGGACCCCAACTGCTCCT	ACAGCCCTGGGCACATTT
MT2	CCGATCTCTCGTCGATCTTCAACC	CAGGAGCAGCAGCTTTTCTTGCAG
GCLC	GCACGGCATCCTCCAGTTCCT	TCGGATGGTTGGGGTTTGTCC
GCLM	GGCTTCGCCTCCGATTGAAGA	TCACACAGCAGGAGGCCAGGT
NQO1	GCATTGGCCACACTCCACCAG	ATGGCCCACAGAGAGGCCAAA
GPx1	TGCTCATTGAGAATGTCGCGTCTC	AGGCATTCCGCAGGAAGGTAAAGA

ALP (alkaline phosphatase); MT1 (metallothionein 1); MT2 (metallothionein 2); GCLC (glutamate-cysteine ligase catalytic subunit); GCLM (glutamate-cysteine ligase modifier subunit); NQO1 (NAD(P)H dehydrogenase, quinone 1); GPx1 (glutathione peroxidase 1)

5. Measurement of intracellular ROS

Generation of intracellular ROS was measured according to the method described by Wang et al.[23] MC3T3E1 cells were cultured on 96-well plates (1 x 10^3 cells/well) and transfected with control siRNA or Nrf1 siRNA. After 24 hr, cells were then incubated in α-MEM containing fluorescent dye 50 μM H2DCF-DA (Invitrogen, Carlsbad, CA) for 15 minutes in the dark, washed thoroughly by PBS and further incubated in α-MEM with or without 10 μg/ml

LPS or 400 μM H_2O_2. The emitted fluorescence was measured by fluorometer (Wallac 1420D fluorometer; PerkinElmer inc., Turku, Finland) with excitation and emission wavelengths at 485 and 535 nm, respectively.

6. Cell proliferation assay

Cells were seeded at 1 x 10^3 cells/ml in 96-well plates and transfected with control siRNA or Nrf1 siRNA using Lipofectamine PLUS reagent. After 48 hr, MTT-based Cell Proliferation Kit I (Roche Applied Science, Mannheim, Germany) was used to measure cell proliferation.

For cellular viability, MC3T3E1 cells transfected with control siRNA or Nrf1 siRNA were treated with LPS for 24 hr. Following treatment, MTT reduction was measured by absorbance at a wavelength of 550 nm.

7. Alkaline phosphatase (ALP) staining

ALP staining was performed to monitor osteoblastic differentiation. Cells were washed with PBS, fixed with 4% paraformaldehyde, and stained for alkaline phosphatase according to the manufacturer's instructions (Sigma, Saint Louis, MO).

8. Micromass culture

Limb buds of E13 embryos were isolated in calcium-/magnesium-free saline (CMF) and digested in 1.2 unit/ml dispase II (Roche) in CMF at 37 ℃ for 25 min. Mesenchymal cells were gently dissociated in 1-2 ml medium to produce a single cell suspension. Cells were suspended at 2 x 10^7 cells/ml in all mixture of 60% culture medium (Nutrient mixture F-12 Ham with 10% fetal calf serum, 1-4 mM-L-glutamine, 1% penicillin streptomycin and 200 μg/ml ascorbic acid with 40% CMF containing 10% newborn calf serum). A single 10 μl drop of this suspension was plated on to each 35 mm tissue culture dish, and incubated with control siRNA or Nrf1 siRNA at 37 ℃ in an atmosphere of 5% CO_2/95%

air in a humidified incubator. After 4 days, cultures were fixed in 4% paraformaldehyde in CMF and stained with Alcian blue to assess the extent of chondrogenesis. The quantity of cartilage was estimated from the amount of dye by measuring the absorbance.

9. Statistical analysis

SPSS version 11.5 for windows (SPSS Inc., Chicago, IL) was used for the statistical analysis. Results are all expressed as the mean ± S.E. and the statistical significance was determined by Students' t-test or the one-way analysis of variance ANOVA with Tukey post hoc test. Significance was defined by a P-value of < 0.05.

III. RESULTS

1. Nrf1 expression after oxidant stimulation in osteoblasts

The expression of Nrf1 mRNA in MC3T3E1 cells using designed primers is shown in Figure 1.

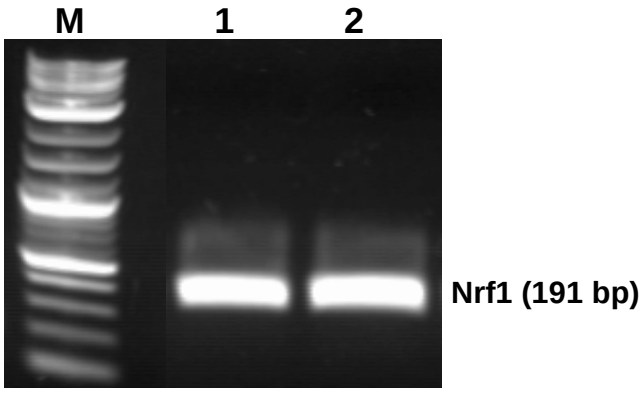

Figure 1. The expression of mRNA for Nrf1 in osteoblastic MC3T3E1 cells. M - DNA size marker, lane 1 and 2 – MC3T3E1 cell line.

This study examined the effect of oxidant treatment on Nrf1 expression in MC3T3E1 cells.

H_2O_2 Treatment of MC3T3E1 cells for 3 hr did not affect the expression of Nrf1 mRNA significantly (Figure 2).

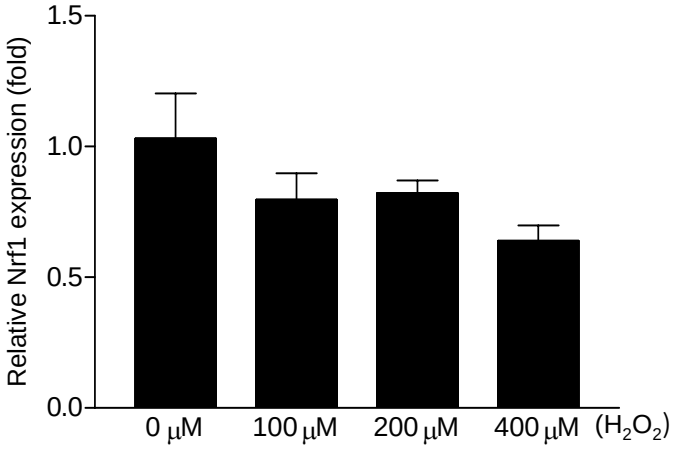

Figure 2. The effect of H_2O_2 on Nrf1 mRNA expression in MC3T3E1 cells. Cells were treated with 0, 100, 200, or 400 μM H_2O_2 for 3 hr. Quantitation of mRNA levels was analyzed by quantitative RT-PCR. The expression level of each mRNA was normalized to the β-actin levels.

To determine the effect of LPS on Nrf1 expression, MC3T3E1 cells were treated with different concentrations of LPS for 24 hr. Nrf1 expression was significantly increased 2.4-fold compared to the control group at 10 μg/ml LPS ($P < 0.05$). A time-dependent response with 10 μg/ml LPS showed a steady increase in Nrf1 expression and a marked increase of Nrf1 expression was observed at 24 hr (Figure 3).

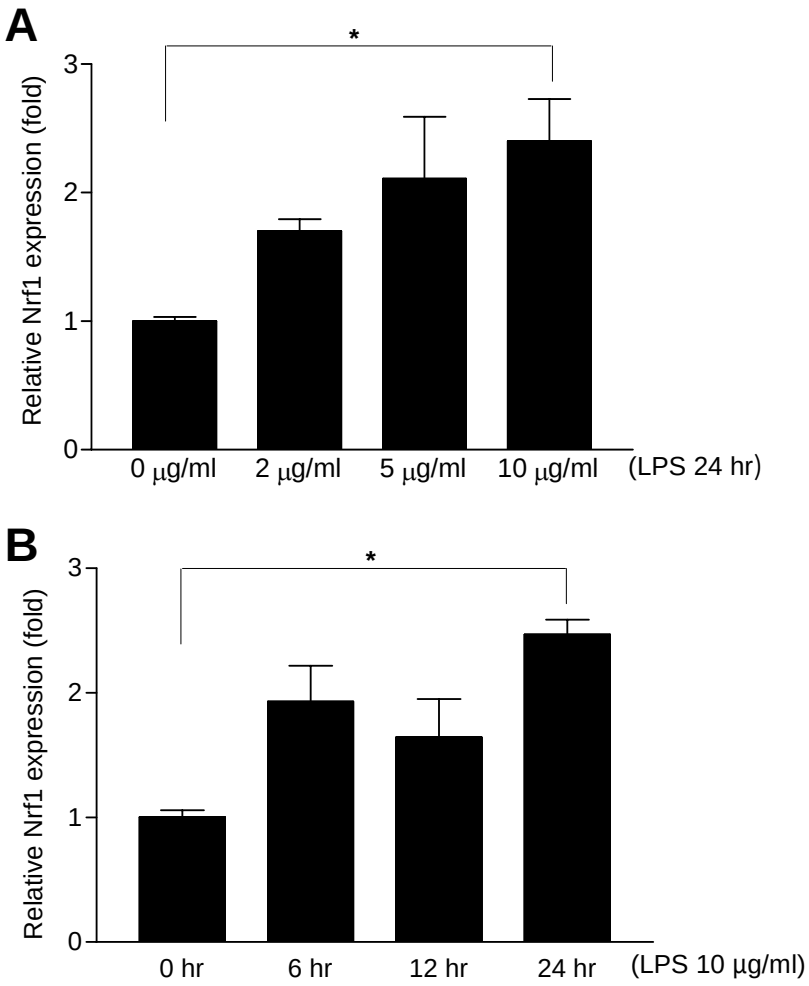

Figure 3. The effect of LPS on Nrf1 mRNA expression in MC3T3E1 cells. (A) Cells were treated with 0, 2, 5, or 10 μg/ml LPS for 24 hr (dose-dependent). (B) Cells were treated with 10 μg/ml LPS for 0, 6, 12, or 24 hr (time-dependent). Quantitation of mRNA levels was analyzed by quantitative RT-PCR. The expression level of each mRNA was normalized to the β-actin levels. *$P < 0.05$ compared with the control group.

2. Effects of Nrf1 knockdown on osteoblast response to oxidative stress

To determine the contribution of Nrf1 to the oxidative stress response in MC3T3E1 cells, specific siRNA for Nrf1 was transfected in MC3T3E1 cells. Transfection with Nrf1 siRNA knocked down expression of Nrf1 by 84% at 20 nM siRNA by quantitative RT-PCR (Figure 4).

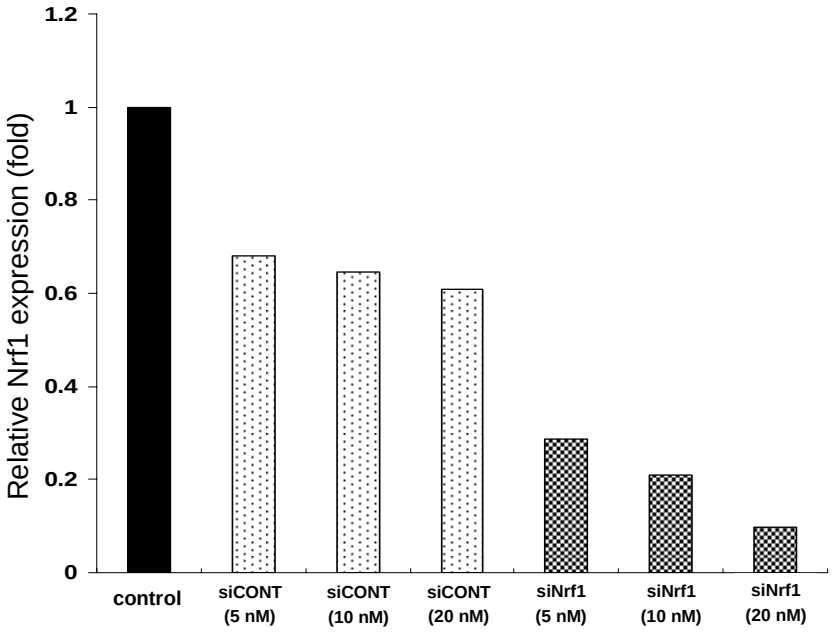

Figure 4. Nrf1 mRNA expression after transient transfection with control siRNA (siCONT) or Nrf1 siRNA (siNrf1) in MC3T3E1 cells. Quantitation of mRNA levels was analyzed by quantitative RT-PCR. The expression level of each mRNA was normalized to the β-actin levels.

To evaluate whether suppression of Nrf1 could alter ROS production, MC3T3E1 cells transfected with control siRNA (siCONT) or Nrf1 siRNA (siNrf1) were treated with LPS (10 μg/ml) or H_2O_2 (400 μM) for 10 minutes, and the ROS generated was analyzed (Figure 5). Quiescent cells (without stimulation) displayed a similar level of ROS in both siCONT and siNrf1 groups. LPS exposure resulted in a significant increase in the amount of ROS by 26% compared with unstimulated cells ($P < 0.05$). Nrf1 siRNA led to an

additional increase of ROS (20%) in the siNrf1 group compared with the siCONT group under LPS stimulation ($P < 0.05$). While the ROS level in H_2O_2-treated cells was approximately 3-fold higher than that in control cells without stimulation ($P < 0.05$), Nrf1 knockdown did not affect ROS production under H_2O_2 stimulation.

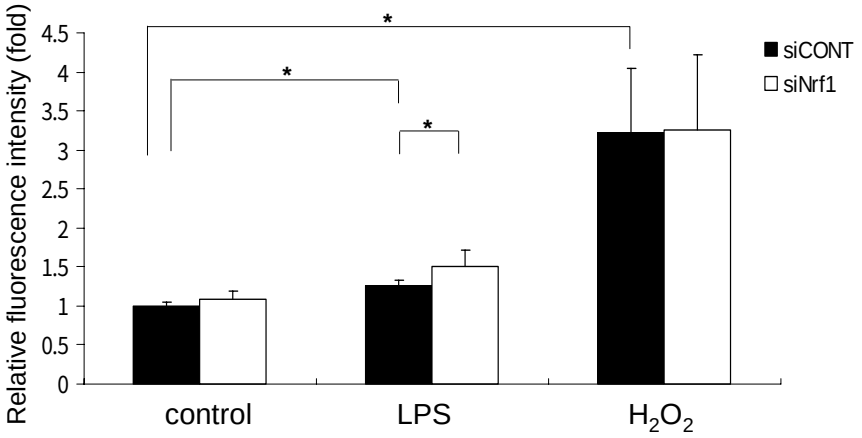

Figure 5. Measurement of ROS with H2DCF-DA in MC3T3E1 cells. Intracellular ROS in the transfectants of control siRNA (siCONT) and Nrf1 siRNA (siNrf1) were compared under control (no stimulation) and stimulations by LPS (10 μg/mL) or H_2O_2 (400 μM) for 10 minutes. *$P < 0.05$ compared with siCONT cells.

This study next examined the effect of mRNA inhibition of Nrf1 on the expression of antioxidant genes against oxidative stress in osteoblasts.

As shown in Figure 6, there was no significant difference in antioxidant gene expression between siCONT and siNrf1 groups under unstimulated conditions. The exposure to LPS significantly increased the level of MT2 compared to untreated controls, and Nrf1 knockdown resulted in a decrease of 48% in MT2 expression under LPS stimulation ($P < 0.05$). In the presence of LPS, Nrf1 siRNA also significantly decreased the expression of GCLC and GPx1 by 41% and 37%, respectively, compared to the siCONT group ($P < 0.05$). However, the

difference in MT1, GCLM, or NQO1 mRNA levels between siCONT and siNrf1 groups was not significant after LPS stimulation.

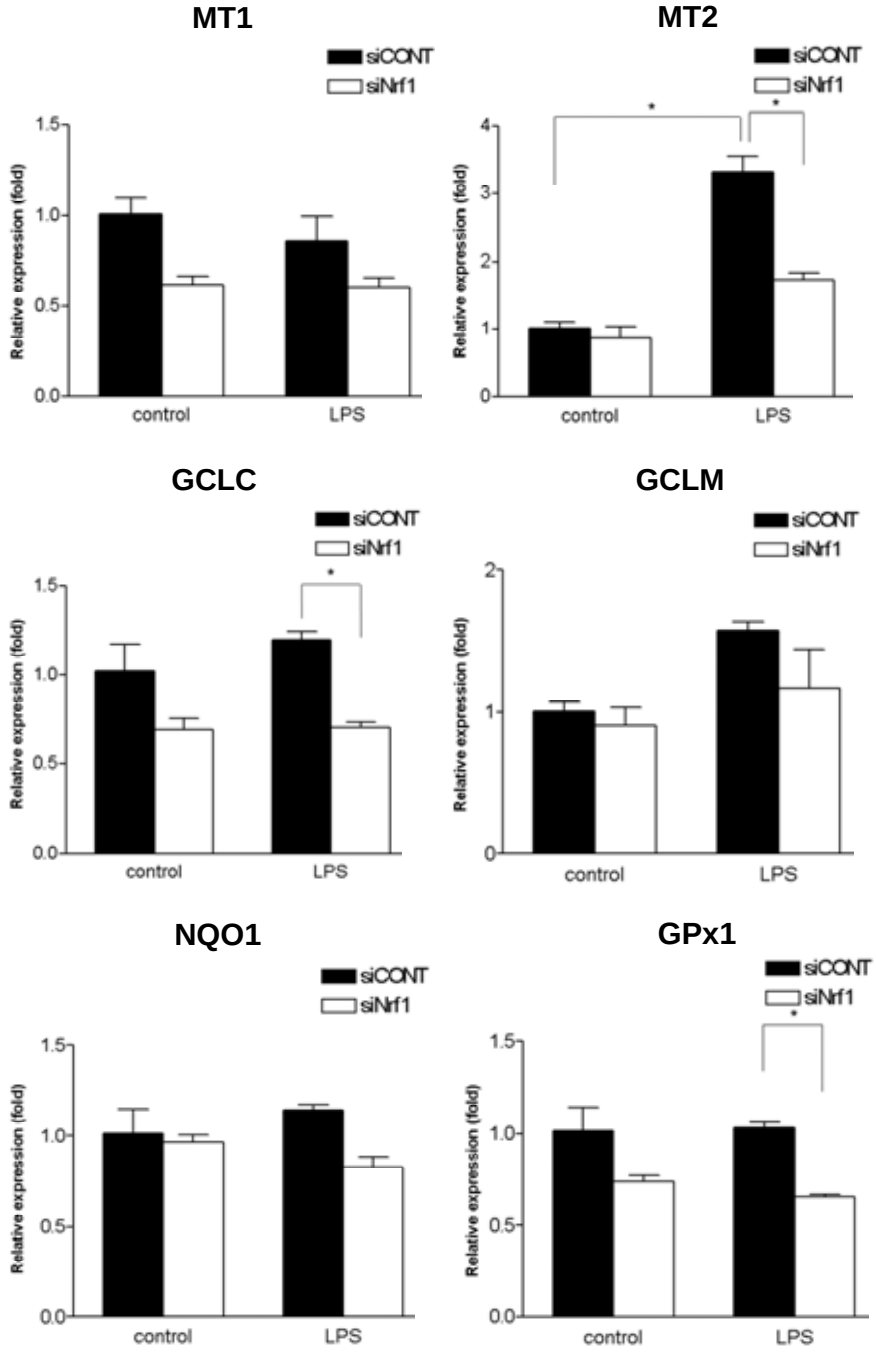

Figure 6. The effect of Nrf1 knockdown on antioxidant gene expression in LPS treated cells. MC3T3E1 cells were transfected with control siRNA (siCONT) or Nrf1 siRNA (siNrf1) followed by 24 hr treatment of 10 μg/ml LPS. Controls received culture medium only. Quantitation of mRNA levels was analyzed by quantitative RT-PCR. The expression level of each mRNA was normalized to the β-actin levels. *$P < 0.05$ compared with the control group.

3. Effects of Nrf1 knockdown on osteoblast proliferation

Under unstimulated conditions, Nrf1 knockdown did not significantly affect osteoblast proliferation (Figure 7A).

In order to measure whether the cell proliferation was decreased by cytotoxicity, each group was incubated with or without 10 μg/ml LPS, and viability was measured using the MTT assay. As seen in Figure 7B, viability level of the siCONT group was comparable to that of the siNrf1 group under LPS stimulation.

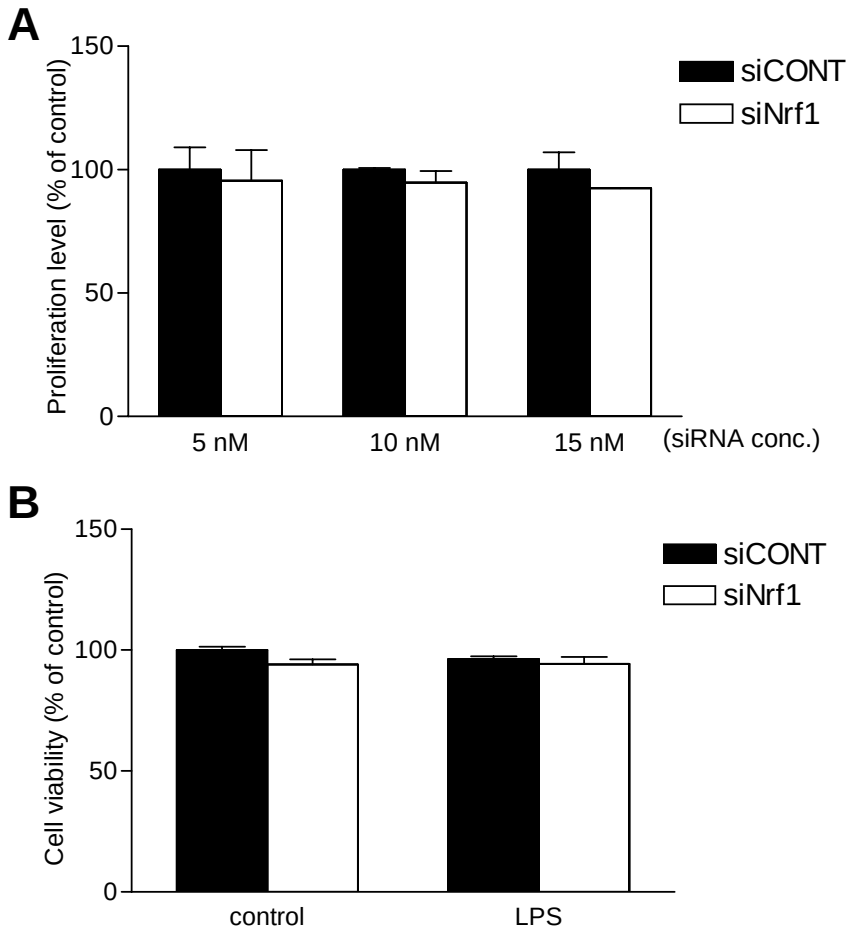

Figure 7. The effect of Nrf1 on the proliferation of MC3T3E1 Cells. (A) MC3T3E1 cells were transfected with control siRNA (siCONT) or Nrf1 siRNA (siNrf1) using Lipofectamin PLUS reagent. After 48 hr culture, MTT assay was done. (B) The transfected cells with control siRNA (siCONT) or Nrf1 siRNA (siNrf1) were treated with 10 μg/ml LPS. After 24 hr, the MTT reduction assay was done.

4. Effects of Nrf1 knockdown on osteoblastic differentiation

To determine whether Nrf1 affects osteoblastic differentiation in MC3T3E1 cells, osteoblast differentiation markers were assessed in cells transfected with

control siRNA or Nrf1 siRNA. The level of ALP mRNA in the siNrf1 group was not different from that in the siCONT group. ALP staining showed no difference between siCONT and siNrf1 groups 14 days after transfection (Figure 8A, B).

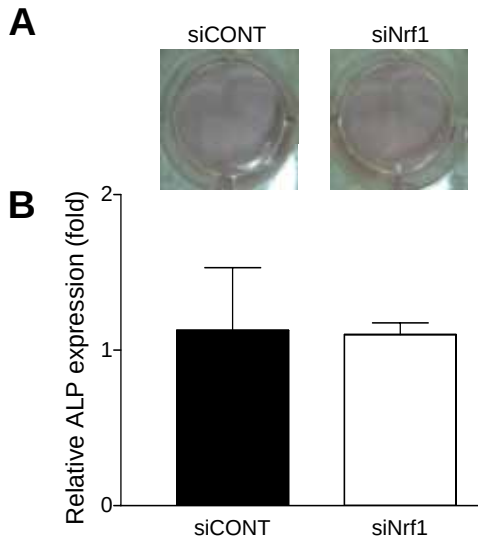

Figure 8. The effect of Nrf1 on the differentiation of MC3T3E1 Cells. After transfection with control siRNA (siCONT) or Nrf1 siRNA (siNrf1), cells were incubated in osteoblast differentiation media containing 50 μg/ml L-ascorbic acid and 10 mM β-glycerophosphate. (A) Representative ALP staining in MC3T3E1 cells 14 days after transfection was shown. (B) Relative expression level of ALP gene was detected by quantitative RT-PCR.

This study further evaluated the effect of Nrf1 on osteoblastic differentiation of MC3T3E1 cells under LPS treatment. Transfectants with control siRNA or Nrf1 siRNA revealed no significant difference in ALP mRNA or ALP staining after osteoblastic differentiation culture with LPS (Figure 9A, B).

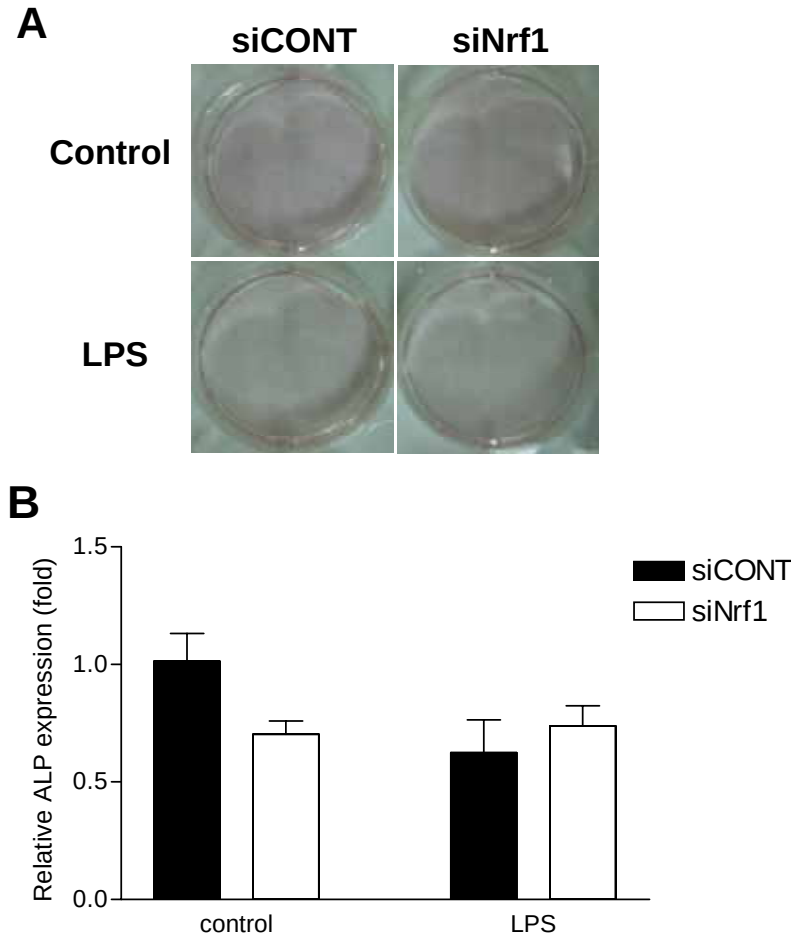

Figure 9. The effect of Nrf1 on the osteoblast differentiation in LPS treated cells. After MC3T3E1 cells were transfected with control siRNA (siCONT) or Nrf1 siRNA (siNrf1), cells were incubated in osteoblast differentiation media containing 50 μg/ml L-ascorbic acid and 10 mM β-glycerophosphate without or with 10 μg/ml LPS. (A) Representative ALP staining was shown 10 days after transfection. (B) Relative expression level of ALP was detected by quantitative RT-PCR 4 days after transfection.

5. Effects of Nrf1 knockdown on chondrogenesis

Nrf1 expression was reported to increase at a high level between 3.5 and 6.5

dpc during development and Nrf1 mutant embryo showed arrested development before 7.5 dpc.[24,25] Therefore, Nrf1 is suggested to have a key role in early development.

Mesenchymal cells derived from the limb buds of 13 dpc embryo were used to evaluate the role of Nrf1 in early skeletal development. Treatment of the micromass cultures with Nrf1 siRNA significantly increased cartilage nodules in comparison to control cultures analyzed on the basis of Alcian blue staining (siCONT group: 0.045 ± 0.003, siNrf1 group: 0.069 ± 0.010, $P < 0.05$)(Figure 10).

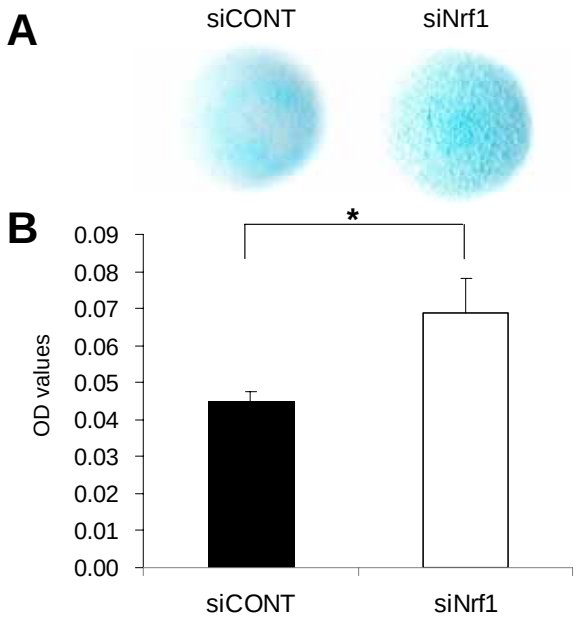

Figure 10. The effect of Nrf1 during chondrogenesis in micromass cultures. During the culture, medium was additionally supplemented with control siRNA (siCONT) or Nrf1 siRNA (siNrf1). (A) Alcian blue staining of whole micromass cultures was shown. (B) OD values for Alcian blue staining were measured. *$P < 0.05$ compared with the siCONT group.

IV. DISCUSSION

Nrf1 is a member of the CNC family of bZIP transcriptional factors and plays an important role in the control of a wide range of genes involved in antioxidants, differentiation and inflammation.[15] Nrf1 shares sequence similarity with Nrf2, another member of the CNC-bZIP family, showing the degree of homology to be 97% in the DNA binding domain and 77% in the leucine zippers region, respectively.[26] However, genetic mutant mice showed different phenotypes – Nrf1 deficiency led to embryonic lethality and Nrf2 knockout mice produced no specific defects.[26,27] These results suggest that Nrf1 and Nrf2 have the functional redundancy and also a crucial difference in their position.[15]

This study demonstrated the change of Nrf1 expression in response to the oxidative stress in osteoblasts. Nrf1 expression was increased by LPS, not H_2O_2 in MC3T3E1 cells. According to the hierarchical oxidative stress model, different redox-sensitive transcription factors are triggered depending on the level of ROS, and Nrf2 is induced at a low oxidative stress level.[28] The present results showed that the level of intracellular ROS was approximately 2.6-fold higher in H_2O_2-treated cells compared to LPS-treated cells. Considering overlapping properties of Nrf1 with that of Nrf2, Nrf1 might sensitively react to LPS, generating less intracellular ROS than H_2O_2 did.

Many studies indicate that induction of Nrf1 tends to vary depending on activators. Nrf1 mRNA and protein were upregulated in the hippocampus damaged by glutamate; meanwhile, Nrf1 was activated by t-butyl hydroquinone (tBHQ) with no change in the amount of Nrf1 protein.[29,30] The increased expression of glutathione peroxidase, one of the antioxidant enzymes, was accompanied by an increase in the cytosolic accumulation of Nrf1, and this reaction was mediated by genistein, not daidzein.[31] The present study confirmed the formation of intracellular ROS induced by LPS was greater in Nrf1 knockdown cells than in control cells. However, reduction of Nrf1 gene did not

influence H_2O_2-induced ROS formation. Taken together, these results suggest that Nrf1 activation is dependent on the type of oxidant.

To my knowledge, this is the first time the role of Nrf1 in the regulation of antioxidant genes in osteoblasts has been evaluated.

In this study, basal levels of antioxidant genes were not changed after suppression of Nrf1 by siRNA in osteoblasts. Under steady state condition, antioxidant genes might be maintained by residual functions of Nrf1.

However, the expressions of GCLC and GPx1 were affected after Nrf1 knockdown under oxidative stress conditions induced by LPS. Among antioxidants, GCLC and GPx1 were reported to be induced in response to overexpression of Nrf1 or increased intracellular accumulation of Nrf1 in previous studies.[18,31]

LPS treatment led to a strong induction of MT2 expression in MC3T3E1 cells, as MT gene is known to be regulated by heavy metals, glucocorticoid hormones, and LPS *in vivo* transcriptionally.[32] The amount of MT2 expression is relatively much higher than MT1, as the ratio of MT1 mRNA: MT2 mRNA was 2:3 ~ 5:7.[32] While mouse MT1 and MT2 genes were regulated only by metals after transfection into HeLa cells *in vitro*,[32] MT2 gene was also induced by LPS in MC3T3E1 cell line and showed stronger signal than MT1 in the present experiment. In addition, functional inhibition of Nrf1 by siRNA in MC3T3E1 cells caused a significant decrease in expression of MT2 but not MT1 in the presence of LPS. The mouse MT1 and MT2 genes are located in close proximity on chromosome 8 and amplified together by heavy metals like cadmium.[32,33] Although the ARE of mouse MT1 gene was reported to be regulated by Nrf1 preferentially,[19] the presence of two MT genes could allow greater flexibility in the regulation of expression depending on the different types of inducers. Therefore, MT2 expression in osteoblasts may be exaggerated after LPS stimulation and dominantly affected by reduction of Nrf1 under oxidative stress in this study.

Since Nrf2 has been shown to regulate GCLM and NQO1,[34,35] down-regulation of GCLM and NQO expression was not apparent in Nrf1 knockdown cells probably due to functional compensation of Nrf2.

The present data that osteoblasts transfected with Nrf1 siRNA did not exhibit reduced proliferation rate are similar to those previously reported.[36,37] Although Nrf1 mutant fibroblasts showed about a 2-fold increase in levels of ROS compared to wild type cells, the viability of Nrf1-/- cells was not different from that of wild type cells.[36,37] However, Nrf1-/- fibroblasts showed reduced viability to the toxic agents such as paraquat or cadmium compared with wild type cells.[16]

In this study, the MTT reduction did not reveal an appreciable change before and after LPS exposure and the suppression of Nrf1 in MC3T3E1 cells. Accumulated data have shown that LPS has variable effects according to the concentration of LPS and types of cells. Whereas low dose (100 ng/ml) of LPS can directly induce cell death or apoptosis in macrophages and vascular endothelial cells, LPS has an anti-apoptotic effect on neutrophils.[38-40] Low concentration (500 ng/ml) of LPS is beneficial for osteoblast proliferation; however, 10 μg/ml LPS does not have a favourable effect on fibroblast proliferation and there is a suppression of cell growth at more than 10 μg/ml concentration of LPS.[41,42] MC3T3E1 cells were treated with 10 μg/ml LPS in this study, which might lead to a neutral effect on the viability of osteoblasts. And I cannot rule out the possibility that a small increase in formation of ROS by LPS is insufficient for a lethal effect on osteoblasts, or that partial redundancy of Nrf2 can compensate for the loss of Nrf1 function in cellular survival and responses to oxidative stress.

The present study then found that the suppression of Nrf1 by siRNA or LPS treatment had no effects on the osteoblastic differentiation in MC3T3E1 cells.

During embryonal development, Nrf1 is expressed at the earliest stage (7 ± 7.5 dpc), differentially distributed in the heart and the presumptive midbrain at 8

dpc, and, again expressed generally throughout the embryo at 8.75 ± 9 dpc.[24] Previous studies established the role of Nrf1 in osteoblast differentiation using BMS from gulonolactone oxidase-deficient mice and from conditional Nrf1 knockout mice.[21,22] These cells are in an earlier developmental stage than MC3T3E1 cells. If Nrf1 have a role in the differentiation in a stage-specific manner, it is possible that MC3T3E1 cells might already escape from the influence of Nrf1 during osteoblastic differentiation. This theory is supported by the present data that mesenchymal cells from E13 limb buds showed a significant difference in formation of cartilage nodules in response to the knockdown of Nrf1 expression. Micromass culture results suggest that Nrf1 might inhibit chondrogenesis or function as a positive mediator for transdifferentiation of chondrocytes into osteoblasts during the process of endochondral ossification. However, further study is needed to demonstrate the precise mechanism of Nrf1 regulating endochondral ossification.

Other explanations for the negative results regarding differentiation in this study are: 1) the remaining amount of Nrf1 might be sufficient to mediate ascorbic acid effects and 2) incomplete suppression of Nrf1-osterix pathway could preserve osteoblastic differentiation while the expression of Nrf1 was knocked down.

In summary, gene expression of the transcription factor Nrf1 was upregulated after LPS exposure in MC3T3E1 cells. Transfection with siRNA against Nrf1 led to increased formation of intracellular ROS and suppressed expression of several antioxidant genes in the presence of LPS in MC3T3E1 cells. Nrf1 contributes to the regulation of antioxidant genes such as MT2, GCLC, and GPx1 in osteoblasts under oxidative stress. Knockdown of Nrf1 expression was not sensitized to MC3T3E1 cells proliferation or differentiation under unstimulated or oxidative stress conditions. However, knockdown of Nrf1 expression showed increased cartilage nodules in embryonal limb bud cells. This *in vitro* study suggests that Nrf1 alone does not have a decisive role in

osteoblast proliferation or differentiation. However, Nrf1 might have a variable effect on cellular differentiation according to the stage of development. Further studies using conditional knockouts of Nrf1 and Nrf2 should be considered to understand the physiologic role of Nrf1 in the oxidative stress defense and development.

V. CONCLUSION

This study demonstrates the role of transcriptional factor Nrf1 in osteoblast response to oxidative stress, and also reveals the effect of Nrf1 on osteoblastic proliferation and differentiation in MC3T3E1 cells.

Nrf1 gene is expressed in MC3T3E1 cells and induced after treatment of MC3T3E1 cells with LPS. Nrf1 mediates expression of antioxidant genes such as MT2, GCLC, and GPx1 under oxidative stress induced by LPS in osteoblasts. However, this *in vitro* study does not show that Nrf1 alone has a critical effect on osteoblast proliferation under unstimulated or stimulated conditions. This study also suggests that Nrf1 is not a decisive factor in osteoblast differentiation, but might have a variable effect on cellular differentiation according to the stage of development. Further study is needed to evaluate the comprehensive mechanisms of the Nrf1-antioxidant pathway in various pathologic conditions.

REFERENCES

1. Banfi G, Iorio EL, Corsi MM. Oxidative stress, free radicals and bone remodeling. Clin Chem Lab Med 2008;46:1550–55.

2. Sheweita SA, Khoshhal KI. Calcium metabolism and oxidative stress in bone fractures: role of antioxidants. Curr Drug Metab 2007;8:519–25.

3. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007;39:44-84.

4. Lyakhovich VV, Vavilin VA, Zenkov NK, Menshchikova EB. Active Defense under Oxidative Stress. The Antioxidant Responsive. Element Biochemistry (Mosc) 2006;71:962-74.

5. Schletter J, Heine H, Ulmer AJ, Rietschel ET. Molecular mechanisms of endotoxin activity. Arch Microbiol 1995;164:383-9.

6. Nair SP, Meghji S, Wilson M, Reddi K, White P, Henderson B. Bacterially induced bone destruction: Mechanisms and misconceptions. Infect Immun 1996;64:2371–80.

7. Kato N, Nakayama Y, Nakajima Y, Samoto H, Saito R, Yamanouchi F et al. Regulation of bone sialoprotein (BSP) gene transcription by lipopolysaccharide. J Cell Biochem 2006;97:368–79.

8. Chan JY, Han XL, Kan YW. Cloning of Nrf1, an NF-E2-related transcription factor, by genetic selection in yeast. Proc Natl Acad Sci U S A

1993;90:11371–5.

9. Kobayashi A, Ito E, Toki T, Kogame K, Takahashi S, Igarashi K et al. Molecular cloning and functional characterization of a new Cap'n' collar family transcription factor Nrf3. J Biol Chem 1999;274:6443-52.

10. Andrews NC, Erdjument-Bromage H, Davidson MB, Tempst P, Orkin SH. Erythroid transcription factor NF-E2 is a haematopoietic-specific basic-leucine zipper protein. Nature 1993;362:722-8.

11. Caterina JJ, Donze D, Sun CW, Ciavatta DJ, Townes TM. Cloning and functional characterization of LCR-F1: a bZIP transcription factor that activates erythroid-specific, human globin gene expression. Nucleic Acids Res 1994;22:2383-91.

12. Ney PA, Andrews NC, Jane SM, Safer B, Purucker ME, Weremowicz S et al. Purification of the human NF-E2 complex: cDNA cloning of the hematopoietic cell-specific subunit and evidence for an associated partner. Mol Cell Biol 1993;13:5604-12.

13. Oyake T, Itoh K, Motohashi H, Hayashi N, Hoshino H, Nishizawa M et al. Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site. Mol Cell Biol 1996;16:6083-95.

14. Motohashi H, Shavit JA, Igarashi K, Yamamoto M, Engel JD. The world according to Maf. Nucleic Acids Res 1997;25:2953-59.

15. Biswas M, Chan JY. Role of Nrf1 in antioxidant response

element-mediated gene expression and beyond. Toxicol Appl Pharmacol 2010;244:16-20.

16. Kwong M, Kan YW, Chan JY. The CNC basic leucine zipper factor, Nrf1, is essential for cell survival in response to oxidative stress-inducing agents. Role for Nrf1 in gamma-gcs(l) and gss expression in mouse fibroblasts. J Biol Chem 1999;274:37491-8.

17. Lee TD, Yang H, Whang J, Lu SC. Cloning and characterization of the human glutathione synthetase 5′-flanking region. Biochem J 2005;390:521-8.

18. Myhrstad MC, Husberg C, Murphy P, Nordström O, Blomhoff R, Moskaug JO et al. TCF11/Nrf1 overexpression increases the intracellular glutathione level and can transactivate the gamma-glutamylcysteine synthetase (GCS) heavy subunit promoter. Biochim Biophys Acta 2001;1517:212-9.

19. Ohtsuji M, Katsuoka F, Kobayashi A, Aburatani H, Hayes JD, Yamamoto M. Nrf1 and Nrf2 play distinct roles in activation of antioxidant response element-dependent genes. J Biol Chem 2008;283:33554-62.

20. Narayanan K, Ramachandran A, Peterson MC, Hao J, Kolsto AB, Friedman AD et al. The CCAAT enhancer-binding protein (C/EBP)β and Nrf1 interact to regulate dentin sialophosphoprotein (DSPP) gene expression during odontoblast differentiation. J Biol Chem 2004;279:45423-32.

21. Xing W, Singgih A, Kapoor A, Alarcon CM, Baylink DJ, Mohan S. Nuclear factor-E2-related factor-1 mediates ascorbic acid induction of

osterix expression via interaction with antioxidant-responsive element in bone cells. J Biol Chem 2007;282:22052-61.

22. Kim J, Xing W, Wergedal J, Chan JY, Mohan S. Targeted disruption of nuclear factor erythroid-derived 2-like 1 in osteoblasts reduces bone size and bone formation in mice. Physiol Genomics 2010;40:100-10.

23. Wang Y, Lou MF. The regulation of NADPH oxidase and its association with cell proliferation in human lens epithelial cells invest. Ophthalmol Vis Sci 2009;50:2291-300.

24. Murphy P, Kolstø A. Expression of the bZIP transcription factor TCF11 and its potential dimerization partners during development. Mech Dev 2000;97:141-8.

25. Farmer SC, Sun CW, Winnier GE, Hogan BL, Townes TM. The bZIP transcription factor LCR-F1 is essential for mesoderm formation in mouse development. Genes Dev 1997;11:786-98.

26. Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the b-globin locus control region. Proc Natl Acad Sci U S A 1994;91:9926-30.

27. Chan JY, Kwong M, Lu R, Chang J, Wang B, Yen TS et al. Targeted disruption of the ubiquitous CNC-bZIP transcription factor, Nrf-1, results in anemia and embryonic lethality in mice. EMBO J 1998;17:1779-87.

28. Gloire G, Legrand-Poels S, Piette J. NF-kB activation by reactive oxygen

species: Fifteen years later. Biochem Pharmacol 2006;72:1493-505.

29. Hertel M, Braun S, Durka S, Alzheimer C, Werner S. Upregulation and activation of the Nrf-1 transcription factor in the lesioned hippocampus. Eur J Neurosci 2002;15:1707-11.

30. Zhnag Y, Lucocq JM, Hayes JD. The Nrf1 CNC/bZIP protein is a nuclear envelope-bound transcription factor that is activated by t-butyl hydroquinone but not by endoplasmic reticulum stressors. Biochem J 2009;418:293–310.

31. Hernandez-Montes E, Pollard SE, Vauzour D, Jofre-Montseny L, Rota C, Rimbach G et al. Activation of glutathione peroxidase via Nrf1 mediates genistein's protection against oxidative endothelial cell injury. Biochem Biophys Res Commun 2006;346:851-9.

32. Searle PF, Davison BL, Stuart GW, Wilkie TM, Norstedt G, Palmiter RD. Regulation, linkage, and sequence of mouse metallothionein I and II genes. Mol Cell Biol 1984;4:1221-30.

33. Beach LR, Palmiter RD. Amplification of the metallothionein-I gene in cadmium-resistant mouse cells. Proc Natl Acad Sci U S A. 1981;78:2110-4.

34. Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y et al. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem 2000;275:16023-9.

35. Moinova HR, Mulcahy RT. Up-regulation of the human gamma-glutamylcysteine synthetase regulatory subunit gene involves

binding of Nrf-2 to an electrophile responsive element. Biochem Biophys Res Commun 1999;261:661-8.

36. Chen L, Kwong M, Lu R, Ginzinger D, Lee C, Leung L et al. Nrf1 is critical for redox balance and survival of liver cells during development. Mol Cell Biol 2003;23:4673-86.

37. Leung L, Kwong M, Hou S, Lee C, Chan JY. Deficiency of the Nrf1 and Nrf2 transcription factors results in early embryonic lethality and severe oxidative stress. J Biol Chem 2003;278:48021-9.

38. Munshi N, Fernandis AZ, Cherla RP, Park IW, Ganju RK. Lipopolysaccharide-induced apoptosis of endothelial cells and its inhibition by vascular endothelial growth factor. J Immunol 2002;168:5860–6.

39. Xaus J, Comalada M, Valledor AF, Lloberas J, López-Soriano F, Argilés JM et al. LPS induces apoptosis in macrophages mostly through the autocrine production of TNF-alpha. Blood 2000;95:3823–31.

40. Feterowski C, Weighardt H, Emmanuilidis K, Hartung T, Holzmann B. Immune protection against septic peritonitis in endotoxin-primed mice is related to reduced neutrophil apoptosis. Eur J Immunol 2001;31:1268–77.

41. Wang LY, Wang HY, Ouyang J, Yu L, Chen B, Qin JQ et al. Low concentration of lipopolysaccharide acts on MC3T3-E1 osteoblasts and induces proliferation via the COX-2-independent NFkappaB pathway. Cell Biochem Funct 2009;27:238-42.

42. Aleo JJ. Stimulation of macromolecular synthesis by endotoxin-treated 3T6 fibroblasts. Experientia 1980;36:546-7.

ABSTRACT (IN KOREAN)

조골 세포에서 산화 스트레스 반응에 대한 nuclear factor-E2-related factor 1의 역할

<지도교수 임 승 길>

연세대학교 대학원 의학과

박 소 영

산화 스트레스와 항산화 효소는 세포의 대사를 조절하며, 조골세포 내에서의 산화-환원 반응에 관여한다. Nuclear factor-E2-related factor 1 (Nrf1, NFE2L1)은 antioxidant response element (ARE) 반응을 매개함으로써 항산화 반응을 유도하고, 세포의 분화 및 염증 반응 등을 조절한다고 알려져 있다. 본 연구는 Nrf1이 산화 스트레스에 반응하는 기능을 조골세포 내에서 확인하고, 기존에 알려졌던 Nrf1의 세포 분화 조절능을 조골세포 계열의 세포인 MC3T3E1 cells에서 알아보고자 하였다.

Nrf1은 전조골세포인 MC3T3E1 cells에서 발현되며, MC3T3E1 cells에 lipopolysaccharide (LPS)를 이용한 산화 스트레스 유도 시에 Nrf1의 발현이 증가하였다. LPS 처리는 MC3T3E1 cells 내에서 활성 산소를 증가시켰으며, Nrf1 knockdown 후 LPS를 처

리하면 활성 산소는 증가하고, metallothionein 2 (MT2), glutamate-cysteine ligase catalytic subunit (GCLC), glutathione peroxidase 1 (GPx1)와 같은 항산화 유전자의 발현은 감소하였다. Nrf1이 MC3T3E1 cells의 증식에 미치는 영향을 살펴보았을 때, Nrf1 knockdown 상태 및 LPS 처리는 MC3T3E1 cells의 증식 또는 생존능에 영향을 주지 않았다. 또한, Nrf1 knockdown은 MC3T3E1 cells의 분화에 의미있는 영향을 미치지 않았다. 하지만, E13 배아로부터 얻은 중간엽 세포를 이용하였을 때, Nrf1은 연골 형성에 관여함을 알 수 있었다.

Nrf1은 산화 스트레스 자극시에 조골세포 내의 항산화 반응을 유도한다. 그러나, Nrf1 단독만으로 조골세포의 증식이나 생존능, 또는 분화에는 결정적인 영향을 미치지 않았다. 하지만, 세포의 분화 단계에 따라서 Nrf1은 골격계의 분화 과정에 대한 역할이 달라질 수 있다. 추후 산화 스트레스와 그에 대응하는 방어 메커니즘이 작용하는 생체내에서 Nrf1이 골조직에 미치는 작용과 그와 관련한 기전을 확인하는 지속적인 연구가 필요할 것으로 사료된다.

핵심되는 말: nuclear factor-E2-related factor 1, Nrf1, 산화 스트레스, 조골세포