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NELL2 Function in the Protection of Cells against Endoplasmic Reticulum Stress

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Continuous intra- and extracellular stresses induce disorder of Ca^{2+} homeostasis and accumulation of unfolded protein in the endoplasmic reticulum (ER), which results in ER stress. Severe long-term ER stress triggers apoptosis signaling pathways, resulting in cell death. Neural epidermal growth factor-like like protein 2 (NELL2) has been reported to be important in protection of cells from cell death-inducing environments. In this study, we investigated the cytoprotective effect of NELL2 in the context of ER stress induced by thapsigargin, a strong ER stress inducer, in Cos7 cells. Overexpression of NELL2 prevented ER stress-mediated apoptosis by decreasing expression of ER stress-induced C/EBP homologous protein (CHOP) and increasing ER chaperones. In this context, expression of anti-apoptotic Bcl-xL was increased by NELL2, whereas NELL2 decreased expression of pro-apoptotic proteins, such as cleaved caspases 3 and 7. This anti-apoptotic effect of NELL2 is likely mediated by extracellular signal-regulated kinase (ERK) signaling, because its inhibitor, U0126, inhibited effects of NELL2 on the expression of anti- and pro-apoptotic proteins and on the protection from ER stress-induced cell death.

INTRODUCTION

Neural epidermal growth factor-like like protein 2 (NELL2) is a secreted glycoprotein that is expressed in neural tissues (Kim et al., 2002; Kuroda and Tanizawa, 1999; Oyasu et al., 2000). NELL2 has several functional domains, such as thrombospondin-like, six epidermal growth factor (EGF)-like, and several von Willebrand factor C-like domains. Characteristically, NELL2 has Ca^{2+}-binding sites in its six EGF-like repeat domains, suggesting a contribution to Ca^{2+}-dependent cellular events (Kuroda et al., 1999; Rao et al., 1995). Previous studies have reported that NELL2 may play multifunctional roles in proliferation, differentiation, and protection of neural cells (Choi et al., 2010; Jeong et al., 2008; Kuroda et al., 1999; Nelson et al., 2002). Among its possible functions, a cell survival-promoting effect has been relatively well studied (Aihara et al., 2003; Choi et al., 2010; Jeong et al., 2008; Munemasa et al, 2012) and is mediated by an intracellular mitogen-activated protein kinase (MAPK) pathway (Aihara et al., 2003; Choi et al., 2010).

In this study, we identified a survival-promoting effect of NELL2 on cells in the setting of endoplasmic reticulum (ER) stress-induced cell death. ER stress is caused by problems with protein folding capacity and control of Ca^{2+} levels in the ER, resulting in the accumulation of unfolded proteins (Boyce and Yuan, 2006; Kaufman, 1999; Kaufman and Malhotra, 2014), which triggers the unfolded protein response (UPR) (Schröder and Kaufman, 2005). The UPR is mediated through three ER transmembrane receptors, including RNA-activated protein kinase (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). In normal cells, all three receptors are maintained in an inactive state through binding with an ER chaperone, binding immunoglobulin protein (BiP, also known as glucose regulated protein of 78 kDa, GRP78). When unfolded proteins accumulate in the ER, BiP dissociates from the three receptors, which leads to their activation and triggers the UPR. The UPR is a pro-survival response that decreases unfolded protein accumulation and reinstates ER function (Schröder and Kaufman, 2005). However, if protein aggregation is constant and excessive, and thus, the stress cannot be resolved, signaling switches from pro-survival to pro-apoptotic. In this state, ER stress-induced apoptosis proceeds through an increase in C/EBP homologous protein (CHOP) expression that is activated by a three ER transmembrane receptor-mediated UPR (Szegezdi et al., 2006). CHOP is a major mediator of ER stress-induced apoptosis (Kadowaki et al., 2004), where it regulates various pro- and anti-apoptotic proteins, such as B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), and Bcl-2-associated death promoter (Bad) (Jing et al., 2012; Johnson et al., 2011). CHOP affects the Bax/Bad system in the mitochondria, resulting in caspase 3 activation and apoptosis (Johnson et al., 2011; Kim et al., 2006; Rao et al., 2004).

In this study, we evaluated whether NELL2 protects cells from ER stress-induced death using a monkey kidney cell line, Cos7, that is well known for the study of NELL2 (Kuroda and Tanizawa, 1999). Using this model, we determined the effect of NELL2 on expression of proteins involved in ER stress-induced cell death.

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MATERIALS AND METHODS

Cell culture and treatment

Cos7 cells were maintained in high glucose Dulbeco's modified Eagle medium (DMEM, Hyclone, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin (Hyclone) under a humidified atmosphere with 5% CO_2 in air at 37°C For the experiments, Cos7 cells were serum-starved for 3 h, followed by treatment with 5 μM thapsigargin (TG, Sigma-Aldrich, USA) and/or U0126 (10 μM, Calbiochem, USA).

Stable transfection of NELL2

To determine the effect of NELL2 on cell survival, we used Cos7 cells stably transfected with a NELL2 expression vector (pcDNA-NELL2) that encodes NELL2 using Lipofectamine/PLUS reagent (Invitrogen Corp., USA), as previously described (Choi et al., 2010). As a control, Cos7 cells were transfected with pcDNA-DEST40 vector (Invitrogen). Cells constitutively expressing the transfected vectors were selected using G-418 (400 μg/ml, Sigma-Aldrich), and their expression of NELL2 was assessed using reverse transcription (RT)-PCR and Western blot.

Viability assay

Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetazolium inner salt (MTS) assay (Meley et al., 2010), which is based on a tetrazolium compound, MTS, and an electron acceptor agent, phenazine methosulfate (PMS, Promega Corp., USA). Briefly, cells were seeded into a 96-well plate at a density of 1×10^4 cells per well in 200 μl of medium, stabilized for growth, and then treated with various concentrations of TG in 100 μl of serum-free medium. After 48 h of incubation with TG at 37°C, 20 μl of MTS/PMS mixture solution was added to the culture medium, and cells were further incubated for 3-4 h at 37°C. Finally, absorption readings were performed at 490 nm using a spectrophotomer.

Trypan blue assay

To further determine the protective effect of NELL2 on TG-induced cell death, Cos7 cells, permanently transfected with NELL2 expression vectors or control vectors, were seeded at 1×10^4 cells per well in six-well plates. After culture in the serum-deprived medium treated with 5 μM TG for the indicated time period, the culture medium was removed and the wells were washed with phosphate buffered saline. After harvesting the cells and brief centrifugation (800 rpm for 3 min) to concentrate the cells, the supernatant was removed and an equal volume of trypan blue solution (0.4% trypan blue in PBS) was added. Total cell number was counted by a hemacytometer and cell survival was estimated by trypan blue exclusion assay (Behl et al., 1995; Yun et al., 2013). The number of live cells was estimated by counting trypan-blue-negative cells. The number of trypan blue-positive cells was divided by the total cell number to estimate the percentage of cell death.

Relative RT-PCR

Total RNA was extracted from Cos7 cells using TRI reagent (Lugen Sci., Korea), and RNA concentration was determined spectrophotometrically. For cDNA synthesis, 1 μg of total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega). The primers used for RT-PCR were as follows: glucose regulated protein of 94 kDa (GRP94) sense primer, 5'-TTCAGGCCCTTCCTGAATTT-3'; antisense primer, 5'-CCTTT-GCATCAGGGTCAATG-3', BiP sense primer, 5'-CTGGCAA-AGACGTCAGGAAA-3'; antisense primer, 5'-TTGGTCATGA-CACCTCCCAC-3', calnexin sense primer, 5'-CCAGACGCA-GAGAAA CCTGA-3'; antisense primer, 5'-AGGATAACCAGG-AACAC-GGG-3'; glyceraldehydes-3-phosphate dehydrogenase (GAPDH) sense primer, 5'-CACTGCCACCCAGAAGACTG-3'; and antisense primer, 5'-ACCCTGTTGCTGTAGCC-AAAT-3'. PCR amplification was performed using 30 cycles at 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s followed by an additional extension at 72°C for 5 min. Amplified PCR products were separated by 1% agarose gel electrophoresis and visualized with ethidium bromide staining. The band densities were analyzed with NIH image software and normalized by comparing them to the densities of control GAPDH bands.

Western blot

Harvested Cos7 cells were homogenized in M-PER lysis buffer (Pierce Chemical Co., USA) containing a protease inhibitor cocktail (Roche, Molecular Diagnostics, Germany) and phosphatase inhibitor cocktail (Roche). Extracted proteins (10-30 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane (Millipore, USA). The membrane was blocked in blocking buffer and incubated overnight at 4°C with antibodies specific to CHOP (1:1000, Santa Cruz Biotech., USA, Catalogue No. sc-793), spliced XBP1 (1:1000, Biolegend, USA, Catalogue No. 619501), BiP (1:2000, Cell Signaling Technology, USA, Catalogue No. 3183), cleaved caspase 3 (1:1000, Cell Signaling Technology, Catalogue No. 9664), cleaved caspase 7 (1:1000, Cell Signaling Technology, Catalogue No. 9491), cleaved Poly (ADP-ribose) polymerase (PARP) (1:1000, Cell Signaling Technology, Catalogue No. 9451), and Bcl-xL (1:1500, Cell Signaling Technology, Catalogue No. 2764). Blots were then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:4000, Santa Cruz Biotech., Catalogue No. sc-2004) for 1 h at room temperature. Immunoreactivity was detected with an enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech., UK). Protein loading was normalized using an anti-β-actin antibody (1:10000, Sigma-Aldrich, Catalogue No. A5441).

Statistical analysis

The results were analyzed with a one-way analysis of variance followed by the Student Neuman-Keuls multiple comparison test for unequal replications. Student's *t*-test was used to compare the two groups.

RESULTS

NELL2 protects Cos7 cells against ER stress-induced death

We first identified the effect of NELL2 on ER stress-induced cell death (Fig. 1). TG inhibits the sarcoplasmic/ER Ca^{2+}-ATPase pump (SERCA), which causes severe ER stress resulting in apoptosis (Kass and Orrenius, 1999). When exposed to TG at higher concentration for 48 h, cells transfected with NELL2 expression vectors revealed significantly higher viability than the pcDNA plasmid transfected control group (CTL) (Fig. 1A). Next, we compared cell survival and death ratios between the NELL2 and CTL groups under TG treatment (5 μM) conditions using a trypan blue exclusion assay (Figs. 1B and 1C). While CTL cells gradually died during the incubation period, significantly more cells remained live in the NELL2 group (Fig. 1B)

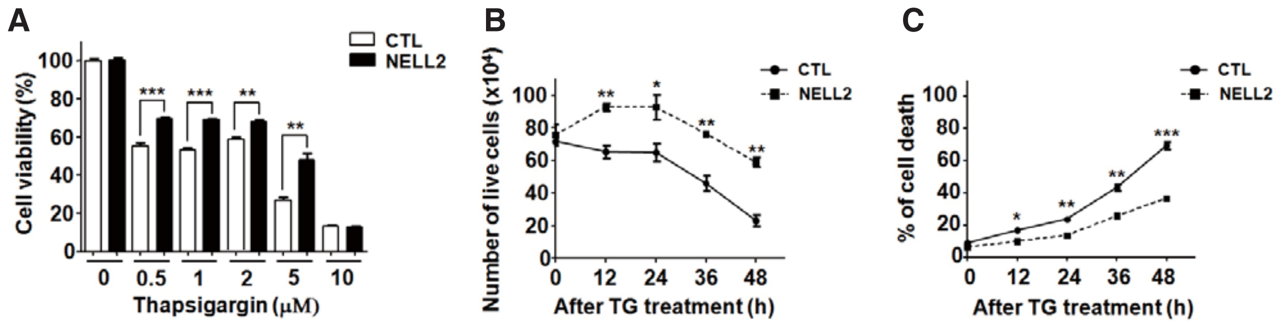

Fig. 1. NELL2 protects Cos7 cells against thapsigargin-induced death. (A) Cell viability was determined after incubation with thapsigargin (TG) using an MTS assay (n = 6-8). Cos7 cells permanently transfected with NELL2 expression vectors (NELL2) or control vectors (CTL) were treated with the indicated concentrations of TG for 48 h. (B, C) Cos7 cells were treated with 5 μM TG for the indicated time period and the number of living cells (B) and percentage of dead cells (C) were counted using a trypan blue exclusion assay (n = 6-8). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. CTL.

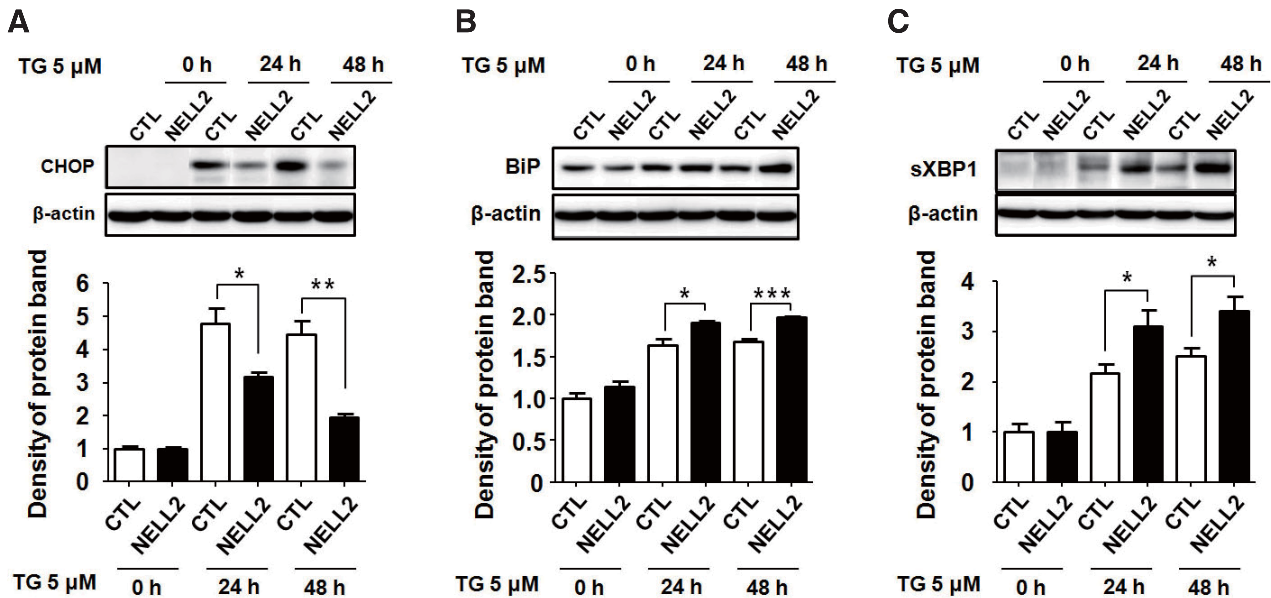

Fig. 2. The effect of NELL2 on ER stress-induced changes in CHOP, BiP, and spliced XBP1 (sXBP) expression. Cos7 cells permanently transfected with NELL2 expression vectors (NELL2) or control vectors (CTL) were treated with 5 μM thapsigargin (TG). After 24 h and 48 h incubations, cells were harvested and protein extracts were analyzed with Western blot for determination of changes in CHOP (A), BiP (B), and sXBP1 (C). Each upper panel reveals representative immunoblots. The lower panel shows calculated data from four repeated Western blots for each protein after normalizing intensity of protein bands with β-actin intensity. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. CTL.

Conversely, fewer cells in the NELL2 group died compared to the CTL group (Fig. 1C). These results suggest that NELL2 protects Cos7 cells from ER stress-induced death.

Effect of NELL2 on the expression of genes responsive to ER stress

We found that NELL2 protected cells from TG-induced ER stress-related death. We then examined whether NELL2 affects expression of a pro-apoptotic protein, CHOP, and proteins involved in the ER stress response, such as BiP and spliced XBP1 (sXBP1), using Western blot analysis. As expected, TG increased CHOP expression, while NELL2 reduced its expression (Fig. 2A). On the contrary, NELL2 increased expression of BiP (Fig. 2B) and sXBP1 (Fig. 2C) in the setting of TG-induced ER stress. These results suggest that NELL2 protected cells from ER stress-induced death through its influence on opposing determinants of ER stress: a cell death inducer, CHOP, and ER stress reducers, such as BiP and sXBP1. Thus, we further confirmed the effect of NELL2 on the expression of mRNA of ER chaperones. NELL2 caused a significant increase in the expression of ER chaperones, such as GRP94 and calnexin, as well as BiP, under conditions of TG-induced ER stress (Supplementary Fig. 1). Moreover, siRNA-mediated knocking-down NELL2 expression resulted in an increased expression of CHOP and a decreased expression of ER chaperones such as GRP94 and BiP in the TG-treated P19 cells (Supplementary

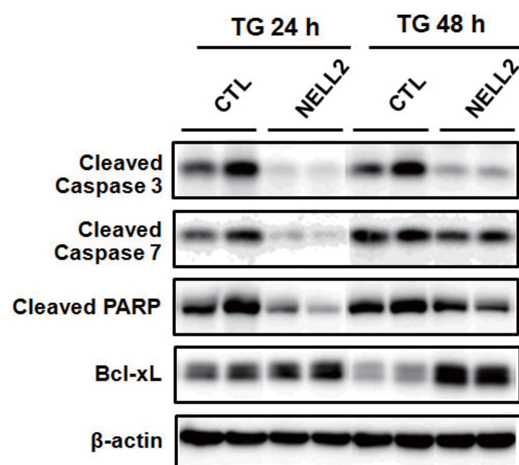

Fig. 3. The effect of NELL2 on ER stress-induced change of pro-apoptotic and anti-apoptotic proteins. Cos7 cells permanently transfected with NELL2 expression vectors (NELL2) or control vectors (CTL) were treated with 5 μM thapsigargin (TG). After 24 h and 48 h incubations, proteins were extracted and Western blot was performed using antibodies against cleaved caspases 3 and 7, cleaved PARP, and BcL-xL. β-actin intensity was used for loading control.

Fig. 2) that natively express NELL2 (Kim et al., 2014), further suggesting that NELL2 is involved in the ER stress by regulating CHOP and ER chaperones.

Effect of NELL2 on the ER stress-induced change of pro- and anti-apoptotic proteins

Previous studies have reported that cell death effectors, such as caspases 3 and 7, are cleaved and activated as part of apoptotic processes under conditions of ER stress (Kim et al., 2006; Rao et al., 2004). Our results revealed that NELL2 decreased cell death in the setting of ER stress by inducing a decrease in CHOP but an increase of BiP and sXBP1. To further delineate NELL2 action in ER stress-induced cell death, we determined the effect of NELL2 on the cleavage of caspases 3 and 7 and PARP under conditions of ER stress. Western blot analyses revealed that NELL2 decreased TG-induced cleavage of caspases 3 and 7 and PARP, whereas it increased expression of Bcl-xL, an anti-apoptotic protein, compared to the CTL group (Fig. 3). These results suggest that NELL2 protects cells from ER stress-induced death both by inhibiting pro-apoptotic proteins and by stimulating an anti-apoptotic protein.

ERK signaling pathway participates in the effect of NELL2 on TG-induced cell death

Our previous study reported that NELL2 elicits a cell survival effect by activating intracellular ERK signaling (Choi et al., 2010). In this study, we addressed whether ERK signaling is also imperative for the survival-promoting action of NELL2 under conditions of ER stress. As shown in Fig. 1, NELL2 increased cell survival after TG treatment, whereas U0126, a mitogen-activated protein kinase kinase (MEK) inhibitor, almost completely abolished this NELL2 effect (Figs. 4A and 4B), suggesting that the cell survival effect of NELL2 requires intracellular ERK/MEK signaling.

To further confirm whether the NELL2 effect on cell survival signaling is ERK dependent, we investigated the effect of

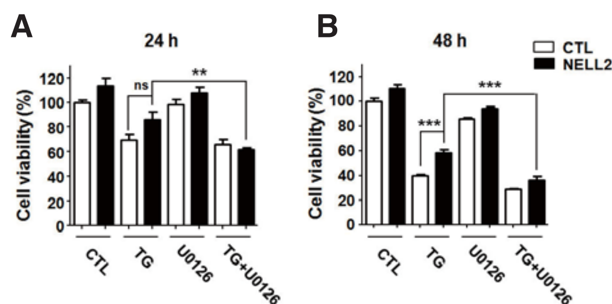

Fig. 4. NELL2 regulates cell viability through ERK signaling. Cos7 cells permanently transfected with NELL2 expression vectors (NELL2) or control vectors (CTL) were treated with 10 μM U0126 (a MEK inhibitor) in the presence or absence of 5 μM thapsigargin (TG) for 24 h (A) and 48 h (B). The percentage of cell viability was measured by MTS assay (n = 6). $^{**}p < 0.01$, $^{***}p < 0.001$. ns, not significant.

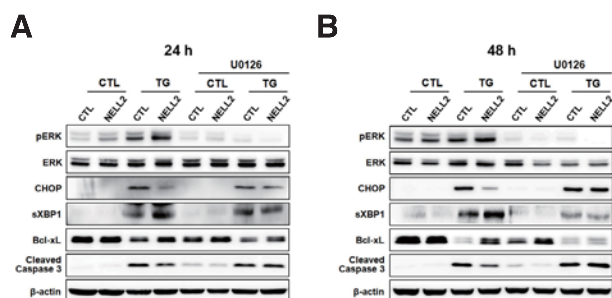

Fig. 5. NELL2 inhibits ER stress-induced cell death signaling through the ERK signaling pathway. Cos7 cells permanently transfected with NELL2 expression vectors (NELL2) or control vectors (CTL) were treated with 10 μM U0126 in the presence or absence of 5 μM thapsigargin (TG) for 24 h (A) and 48 h (B). Representative Western blots revealed the effect of U0126 on ERK phosphorylation (pERK) and expression of CHOP, spliced XBP1 (sXBP1) and Bcl-xL, and cleaved caspase 3. Experiments were repeated twice and resulted in the same pattern of changes.

U0126 on NELL2-induced changes in proteins involved in ER stress-induced cell death. In both CTL and TG-treated cells, NELL2 increased phosphorylated ERK (pERK), an effect that completely disappeared after treatment with U0126 (Figs. 5A and 5B). U0126 inhibited the opposing effects of NELL2, which included decreases in CHOP and cleaved caspase 3 and increases in sXBP1 and Bcl-xL after treatment with TG. These data together suggest that NELL2 affects the ER stress-induced apoptotic process by acting through intracellular ERK signaling.

DISCUSSION

Previous research has shown that NELL2 protects hippocampal neuroprogenitor, HiB5, cells from death induced by cytotoxic factors, such as amyloid β (Choi et al., 2010). In this study, we found that NELL2 played an important role in the protection of Cos7 cells from ER stress-induced death. Under conditions of ER stress, NELL2 contributed to the protection of cells from

ER stress-induced death, by inhibiting expression of CHOP and cleavage of caspases 3 and 7, and by stimulating expression of sXBP1 and ER chaperones, such as BiP and GRP94. An intracellular ERK signaling pathway likely mediates this survival-promoting effect of NELL2, because an ERK inhibitor abolished the NELL2 effect on cell survival.

The ER serves many important intracellular functions, such as protein folding and storage of Ca^{2+}. ER stress is activated in response to an accumulation of misfolded proteins and changes in ionic conditions of the ER lumen (Schröder and Kaufman, 2005). Sustained and excessive ER stress leads to ER stress-induced apoptosis that is facilitated by an increase in CHOP expression. CHOP is expressed at low levels under normal conditions (Marciniak et al., 2004; Puthalakath et al., 2007). Overexpression of CHOP induces apoptosis, while CHOP deficiency can protect cells from ER stress-induced apoptosis (Marciniak et al., 2004; Wang et al., 1996), indicating that CHOP is an important signaling molecule in the ER stress-induced apoptotic pathway. Mitochondrial dysfunction also plays a role in ER stress-induced cell death (Puthalakath et al., 2007; Sano and Reed, 2013). ER stress regulates apoptotic proteins localized on the mitochondrial membrane, such as Bcl-2 family members (Breckenridge et al., 2003). The Bcl-2 family consists of anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, and pro-apoptotic proteins, such as Bax. The balance between anti- and pro-apoptotic proteins is important for maintaining normal mitochondrial function as well as cell survival (Szegezdi et al., 2006).

Previous studies have shown that both anti- and pro-apoptotic proteins are regulated by CHOP during ER stress (McCullough et al., 2001; Puthalakath et al., 2007). CHOP down-regulates expression of Bcl-2 and Bcl-xL but up-regulates expression of Bax. Our results showed that NELL2 inhibited CHOP but activated Bcl-xL. Bcl-xL inhibits the cytochrome c-initiated caspase cascade and decreases level of cleaved PARP in the apoptotic process. Moreover, Bcl-2 and Bcl-xL inhibit cell death by inhibiting release of cytochrome c (Szegezdi et al., 2006). PARP is a member of a protein family involved in a number of cellular processes, such as DNA repair and programmed cell death, and plays a role in protection of cells from death (Malanga and Althaus, 2005). PARP detects single-strand DNA breaks (SSB) and recruits the enzymatic machinery involved in SSB repair (Mangerich and Bürkle, 2011). PARP is cleaved and inactivated by activated (cleaved) caspase-3 during cell death (Boulares et al., 1999). Taken together, CHOP plays a pivotal role in the apoptotic process by regulating the Bcl-2 family. In this regard, NELL2 may protect cells from ER stress-induced apoptosis by suppressing CHOP. CHOP may be, at least in part, responsible for the NELL2-induced changes in expression of proteins involved in the ER stress-induced apoptosis that were observed in this study, including a decrease in cleaved caspases 3 and 7 and cleaved PARP and an increase of Bcl-xL. However, elucidation of the detailed mechanisms of NELL2 action on the ER stress-induced apoptotic process requires further study.

NELL2 is mostly secreted into culture medium from transfected Cos7 and HiB5 cells (Jeong et al., 2008; Kuroda et al., 1999). The released NELL2 stimulates the intracellular ERK pathway through an unknown receptor (Aihara et al., 2003; Choi et al., 2010). The activated ERK stimulates cell survival and differentiation (Choi et al., 2010; Kim et al., 2014). ERK 1/2 and Akt signaling pathways have been shown to prevent ER stress-induced cell death (Hu et al., 2004; Hung et al., 2003; Tian et al., 2011). ERK activation inhibits apoptosis by suppressing function and expression of pro-apoptotic proteins, such as caspases 3 and 9 (Allan et al., 2003). ERK 1/2 can also promote cell survival by increasing the expression of anti-apoptotic proteins, such as Bcl-2 family members, or by increasing the expression and activity of DNA repair proteins (Boucher et al., 2000; Lu and Xu, 2006). Moreover, ERK 1/2 modulates ER stress-induced cell death by suppressing CHOP expression (Lee et al., 2012). Our previous study showed that NELL2 promotes hippocampal neuroprogenitor, HiB5, cell survival by stimulating intracellular ERK signaling (Choi et al., 2010). In this study, we further found that an ERK inhibitor attenuated the NELL2-induced increase in cell survival under conditions of ER stress. Taken together, these results suggest that NELL2-induced ERK signaling is responsible for the survival promoting effect of NELL2 in the context of ER stress through control of proteins involved in cell death and survival, such as CHOP and the Bcl-2 family.

During the ER stress response, ER chaperones promote protein folding and trafficking by preventing mis-folding and aggregation (Voisine et al., 2010). In this study, we found that NELL2 increased expression of sXBP1 and ER chaperones, such as GRP94, BiP, and calnexin. A recent study suggested that ERK signaling is required for activation of IRE1 and ATF6 pathways and sXBP1 in melanoma cells undergoing ER stress and is important for attenuation of ER stress (Tay et al., 2014). Therefore, NELL2 may regulate sXBP1 and BiP through the ERK signaling pathway. However, further study is required to clarify this process.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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