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FGF-2 inhibits TNF-α mediated apoptosis through up-regulation of Bcl2-A1 and Bcl-xL in ATDC5 cells

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FGF-2 is involved in cell survival, proliferation, apoptosis, and angiogenesis in a wide variety of cells. FRGRs, PI3K and MAP kinases are well known mediators of FGF signaling. Despite its known roles during many developmental processes, including osteogenesis, there are few known targets of FGF-2. In the present study, we identified Bcl2-A1 and Bcl-xL as two prominent targets involved in promoting cell survival. Pretreatment of ATDC5 cells with FGF-2 increased cell survival, while siRNAs specific for Bcl2-A1 and Bcl-xL compromised the anti-apoptotic effect of FGF-2, sensitized the cells to apoptosis triggered by TNF-α. Chemical inhibition of FGFR, NFkB, and PI3K activity by PD173074, pyrrolidine dithiocarbamate, and LY294002 respectively abrogated the FGF-2-mediated induction of Bcl2-A1 and Bcl-xL expression. Taken together, our data demonstrate that a subset of Bcl2 family proteins are the targets of FGF-2 signaling that promotes the survival of ATDC5 cells. [BMB reports 2012; 45(5): 287-292]

INTRODUCTION

During the human life cycle, FGFs play ubiquitous roles, commencing at germ cell maturation (1, 2) and continuing throughout embryonic development (3, 4) and into adulthood (5).

FGFs activate FGF receptors to initiate signaling pathways including MAPK, PI3K-AKT, and PLC-γ, promoting survival of various cells (6-8) or inhibiting the proliferation of cancer and primary cells (9, 10). Numerous studies identified the signaling components and pathways of FGFs in diverse cellular contexts, but there is a lack of information regarding which anti- or pro-apoptotic target genes are modulated at the transcriptional level.

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BCL-2 family members have been classically grouped into three classes. One class, which includes Bcl-2, Bcl-xL and Bcl-A1, inhibits apoptosis, whereas the second class (BAX) promotes apoptosis. The third class of BH3-only proteins includes BAD and BID promotes apoptosis (11). Bcl2-A1 was found to be transiently up-regulated in lymphocytes by various stimuli including GM-CSF, CD154, CD40, phorbol ester, TNF and IL-1 (12-14).

ATDC5 is a murine chondrocytic cell line widely used as a monolayer culture system to study chondrogenic differentiation (15) and our previous study demonstrated that they are well responsive to stimulation with FGF-2 to modulate the expression of several genes (16).

In the present study, we investigated FGF-2-inducible genes that may promote the survival of ATDC5 chondroprogenitor cells. Our data indicate that *Bcl2-A1* and *Bcl-xL* are major target genes whose expression and concomitant mitochondrial localization are up-regulated by FGF-2, thorough which block TNF-α mediated cell death in a nuclear factor-kappa B (NF-kB)- and PI3K-dependent manner.

RESULTS

FGF-2 increases the level of Bcl-xL and Bcl2-A1 transcripts

Various concentrations of FGF-2 were added to the normal culture media for 6 days and the cells were stained with crystal violet. FGF-2 appeared to protect the ATDC5 cells from cell death (Fig. 1A). This finding was supported by MTT assay, which revealed the pro survival effect of FGF-2 as shown in Fig. 1B. To identify transcriptional target(s) of FGF-2, total mRNA was extracted from cells treated with FGF-2 for Illumina DNA microarray analysis. Among the 14,000 genes, Bcl2-A1 gene (a, b, and d isoforms) was the most dramatically up-regulated for 25-58 folds by FGF-2. RT-PCR analysis using gene-specific primers confirmed the results from the DNA microarray (data not shown). To further substantiate this finding, an RPA assay was performed. Among the seven genes included in the assay kit, Bfl1 (human homolog of Bcl2-A1) and Bcl-xL/S were prominently up-regulated in the FGF-2-treated

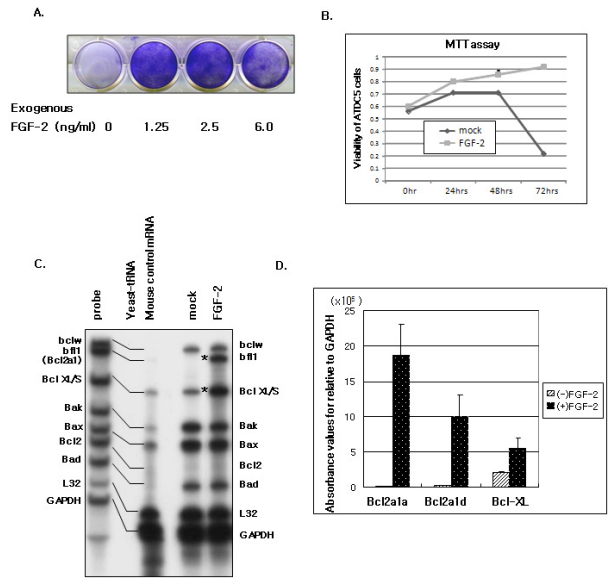

Fig. 1. FGF-2 increases the expression of Bcl-xL and Bcl2-A1 transcripts. (A) ATDC5 cells were grown in the normal media before being treated with 0, 1.25, 2.5, and 6.0 ng/ml FGF-2 for 6 days before staining with crystal violet to visualize the cells in the plate. (B) ATDC5 cells were treated with PBS or FGF-2 up to 72 hours before MTT assay. (C) ATDC5 cells were treated with 5 ng/ml FGF-2 for 1 day and total RNA was subjected to RNase Protection Assay using the mAPO-2 probe set. The asterisks indicate genes that were up-regulated in the FGF-2-treated cells. (D) cDNA of ATDC5 cells treated with FGF-2 (5 ng/ml) was subjected to real-time PCR using primers specific for GAPDH, Bcl2-A1a, Bcl2-A1d, and Bcl-xL.

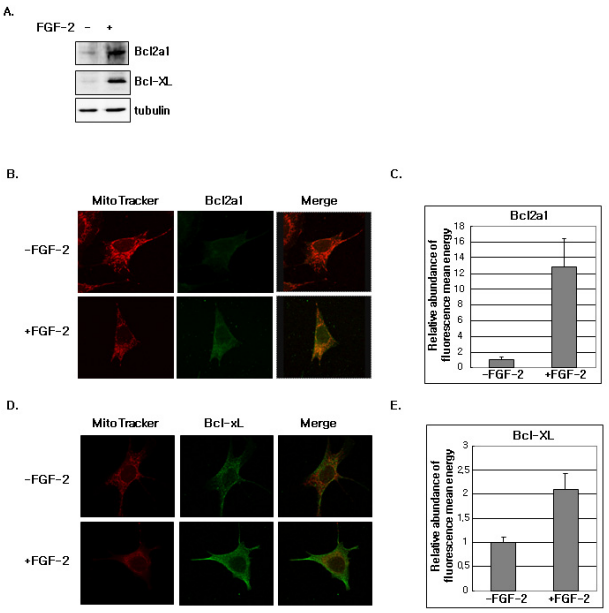

Fig. 2. FGF-2 increases the localization of Bcl2-A1 and Bcl-xL proteins in the mitochondrial compartment. (A) Proteins were extracted from ATDC5 cells after incubation with/without 5 ng/ml FGF-2 for 48 hours. Protein expression was examined by western blotting using antibodies specific for Bcl2-A1, Bcl-xL, or tubulin. (B, D) After incubation with/without FGF-2 (5 ng/ml) for 48 hours, ATDC5 cells were fixed and intracellular localization of the two Bcl2 family proteins was visualized by immunostaining with anti-Bcl2-A1 or anti-Bcl-xL antibody and MitoTracker (B, D). (C, E) Densitometric measurements are presented on the Y-axis as the relative mean fluorescence intensity of the total Bcl2-A1 or Bcl-xL signal.

cells (Fig. 1C). Real time PCR analysis showed that two isoforms of Bcl2-A are clearly induced 10- to 20-folds by FGF-2, compared with 2-fold induction of Bcl-xL (Fig. 1D).

FGF-2 increases the localization of Bcl2-A1 and Bcl-xL proteins in the mitochondrial compartment

ATDC5 cells were treated with 5 ng/ml FGF-2 for 48 hr before western blot analysis with antibodies against Bcl2-A1 or Bcl-xL. Compared to the PBS control, the expression of Bcl2-A1 and Bcl-xL proteins was enhanced by FGF-2 treatment (Fig. 2A). Subsequently, we investigated their intracellular localization using MitoTracker and anti-Bcl2-A1 or anti-Bcl-xL antibodies. As shown in Fig. 2B and C, addition of FGF-2 increased the mitochondrial signal of Bcl2-A1 by 12-folds, Bcl-xL by 2 folds (Fig. 2D and E).

Bcl2-A1 and Bcl-xL are required for protection of ATDC5 cells from TNF-α mediated cell death

To evaluate the significance of the FGF-2 mediated induction of Bcl2-A1 and Bcl-xL in cell survival, ATDC5 cells were treated with 5 ng/ml FGF-2 and/or TNF-α for 24 hours before FACS analysis. Approximately 10% and 2% of PBS and FGF-2-treated ATDC5 cells respectively were present in the sub-G1 population, while TNF-α treatment increased the sub-G1 population 3- folds compared with the PBS (Fig. 3A). Using lipophilic dye JC-1 (17), we showed that, compared with the PBS treated control, TNF-α induced alterations in the mitochondrial membrane potential but this effect was abrogated by FGF-2 (Fig. 3B). In the following study, the expression of Bcl2-A1 and Bcl-xL genes was inhibited by siRNAs (Fig. 3C and D). Compared with cells treated with scrambled control siRNA, ATDC5 cells treated with siRNAs specific for Bcl2-A1 and Bcl-xL were 3- and 6-folds more vulnerable to TNF-α mediated cell death respectively. This suggests that the anti-apoptotic effect of FGF-2 is mediated through up-regulation of Bcl2-A1 and Bcl-xL expression in ATDC5 cells.

NF-kB is a transcriptional mediator of FGF-2 for expression of Bcl2-A1 and Bcl-xL genes

To elucidate which of the FGF-2 signaling pathways are involved in the transcriptional induction of Bcl2-A1 and/or Bcl-xL genes, chemical inhibitors were added individually to

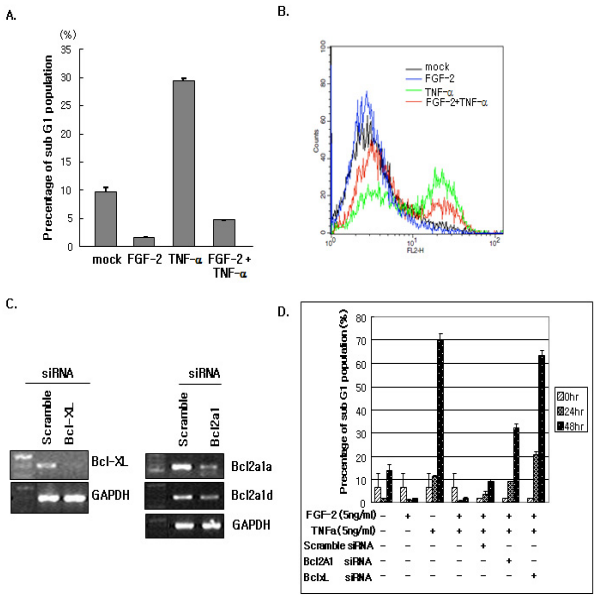

Fig. 3. Bcl2-A1 and Bcl-xL are required for protection of ATDC5 cells from TNF-α mediated cell death. (A) ATDC5 cells were incubated with PBS or 5 ng/ml FGF-2 and/or 5 ng/ml TNF-α for 1 day, followed by staining with propidium iodide for FACS analysis. (B) ATDC5 cells were incubated with 5 ng/ml FGF-2 and/or 5 ng/ml TNF-α before staining with 50 nM cationic cyanine dye DiOC2 (3) to allow flow cytometric measurement of the membrane potential of mitochondria. (C) ATDC5 cells were transfected with scrambled, Bcl-xL, or Bcl2-A1 siRNA and the level of gene silencing was determined by RT-PCR using gene-specific primers. (D) Cells were transfected overnight with scrambled, Bcl-xL, or Bcl2-A1 siRNAs before incubation with 5 ng/ml FGF-2 and/or TNF-α for 0, 24, and 48 hours. Cells were stained with propidium iodide and analyzed by FACS to measure the sub-G1 apoptotic population.

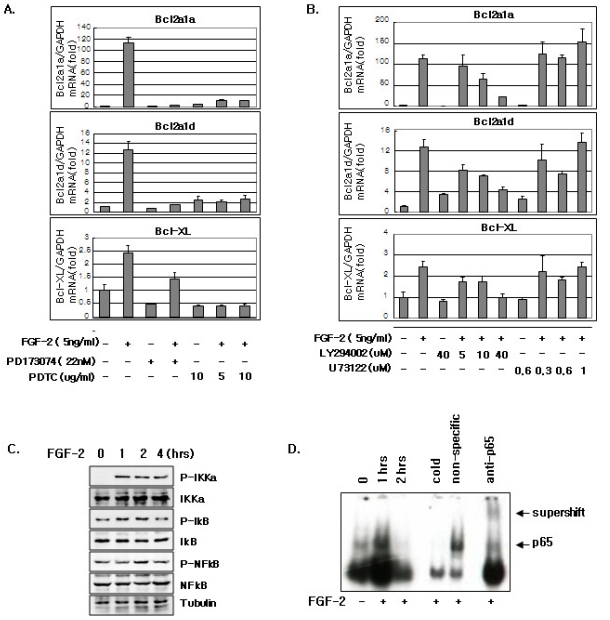

Fig. 4. NF-kB is a transcriptional mediator of FGF-2 that induces the expression of Bcl2-A1 and Bcl-xL genes. (A) ATDC5 cells were pre-incubated with or without PD173074 (22 nM) and PDTC (5, 10 μg/ml) for 1 h, and then treated with FGF-2 (5 ng/ml) for 8 hours. Real-time PCR analysis was conducted using primers specific for Bcl2-Ala, Bcl2-A1d, Bcl-xL, and GAPDH as an internal control. (B) ATDC5 cells were pre-incubated with or without LY294002 (5, 10, 40 μM) and U73122 (0.3, 0.6, 1 μM) for 1 hour prior to real-time PCR analysis as described above. (C) Proteins were extracted from ATDC5 cells after treatment with FGF-2 for 0, 1, 2, and 4 hours and subjected to western blotting using antibodies specific for P- IKKα IKKα, P-IkB-α, IkB-α, P-NF-kB, NF-kB, and tubulin. (D) Proteins from the nuclear fraction of ATDC5 cells stimulated with FGF-2 for 0, 1, and 2 hr were incubated with p32-labeled NF-kB P65 consensus probe and subjected to EMSA. "Cold" indicates EMSA performed with cold competitor p65 probe. For the supershift assay, anti-p65 antibody was added to the nuclear fraction before EMSA analysis.

the growth media of ATDC5 cells 1 hour before addition of FGF-2. RT-PCR analysis revealed that the transcript levels of both Bcl2-A1 and Bcl-xL genes were decreased by PDTC (NF-kB inhibitor), while PD173074 (FGFR inhibitor) inhibited the transcription activity of Bcl2-A1 gene and, to a lesser extent, Bcl-xL gene (Fig. 4A). Neither LY294002 (PI3K inhibitor) nor U73122 (PLC inhibitor) had a significant effect (Fig. 4B). Based on these findings, NF-kB activity was investigated by western blotting using phospho-specific antibodies and by EMSA. FGF-2 treatment markedly increased the phosphorylation of IKK and to a less extent, phosphorylation of IkB (Fig. 4C). In EMSA, the FGF-2 mediated effect appeared to be transient, since there was an increased binding activity of p65 to the probe within 1 hour, but not at 2 hours after stimulation. Addition of anti-p65 antibody to the reaction mixture resulted in the appearance of a supershifted band (Fig. 4D).

DISCUSSION

Despite its apparent role in the balance between cell survival and cell death, there is a lack of detailed information on the target gene(s) or protein(s) of FGF involved in this process.

The aim of the present study was to identify transcriptional targets of FGF-2 that may play a role in the survival process of ATDC5 chondroprogenitor cells.

DNA microarray analysis revealed that Bcl2-A1, an anti-apoptotic Bcl2 family gene, was up-regulated at the transcriptional level. Subsequently, Bcl2-A1 was consistently shown to be up-regulated at the transcriptional and translational levels by real-time PCR, RPA, western blot, and confocal analysis. The expression profile of Bcl2-A1a seemed to be slightly different from that of Bcl2-A1d in that the expression of the former was more tightly regulated than that of the latter.

There appeared to be a low basal level of Bcl-xL gene expression that could be further up-regulated by FGF-2 as shown in Fig. 1E, F and Fig. 2. Interestingly, the level of Bcl-xL induction was found to be higher in the RPA assay than by RT-PCR. In contrast, Bcl2 expression levels were unaffected by FGF-2 treatment. DNA microarray and RPA assays revealed that the mRNA levels of other Bcl2 family genes were similarly unaltered.

The expression of Bcl2-A1 and Bcl-xL proteins increased 10- and 2-folds respectively following FGF-2 treatment. Subsequent confocal imaging of endogenous target proteins revealed that the most of the Bcl2-A1signal was present in mitochondrial compartments together with the MitoTracker signal. The signal of Bcl-xL around the nucleus merged well with the MitoTracker signal, but there was an unexpected signal at the cell periphery that appeared to be distinct from MitoTracker. Recently, FKBP38 was shown to play a role in directing Bcl2 and Bcl-xL proteins to either mitochondria or the cell periphery, including the plasma membrane, depending on the presence of the transmembrane domain (TM) of FKBP38. Cells with a TM deletion of FKBP38 and localization of Bcl-xL at the cell periphery were more sensitive to cell death (18,19). However, it is not clear whether the aberrant localization of Bcl-xL observed in our study is related to this finding and the sensitization of ATDC5 cells to cell death. Although the degree of induction of Bcl2-A1 appears greater than that of Bcl-xL, the relative amount of Bcl-xL transcripts and protein seems larger than that of Bcl2-A1, and it is possible that Bcl-xL plays a larger role in the protection from cell death. Along this line, Bcl-xL is known to be 10 times more effective than Bcl2 in protecting breast cancer cells from apoptotic stimuli including etopside, ceramide, and TNF-α (20).

While FGF-2 decreased the size of the cell population undergoing apoptosis, TNF-α increased it by 3-folds compared to PBS treated cells. On the other hand, in the presence of FGF-2, TNF-α mediated cell death was reduced to a level even lower than that of the PBS control and increasing the dose of TNF-α did not abolish the effect of FGF-2. TNF-α treatment did not interfere with the expression of Bcl2-A1 and Bcl-xL that is otherwise induced by FGF-2, in fact it slightly induced it. Gene-specific siRNAs against Bcl-xL and Bcl2-A1 confirmed that these genes are important targets of the FGF-2 signaling pathway in controlling the cell survival process. Furthermore, concurrent up-regulation of Bcl-xL and Bcl2-A1 was reported to induce approximately 1000-fold resistance to ABT-737 in chronic lymphocytic leukemia (CLL) (20) and by the same token, FGF-2-mediated induction of both genes might contribute to antiapoptotic activity.

The activation of Bcl2-A1 and/or Bcl-xL is involved in NF-kB signaling in CLL (21), CD40 activation (22), and etopside- or TNF-induced cell death (23, 24). In the present study, we tested whether the induction of these genes requires NF-kB and/or other signaling proteins. While the induction of Bcl2-A1 and Bcl2-xL expression was respectively fully and partially inhibited by the FGFR inhibitor PD173074, both genes were fully inhibited by NF-kB inhibitor. FGF-2 appeared to activate the NF-kB signaling cascade by significantly increasing the phosphorylation of IKK-α and slightly increasing that of IkB. Cytoplasmic localization of the p65 subunit in the NF-kB complex decreased and its nuclear localization increased in response to FGF-2 stimulation. In addition, FGF-2 stimulated the binding of p65 subunit to radiolabeled NF-kB-specific probe and anti-p65 antibody caused a supershift of the probe-NF-kB complex. Interestingly, NF-kB appeared to have a short window of probe binding that lasted for 1 hour, then disappeared within 2 hours after FGF-2 stimulation. Inhibition of PLC-γ by U73122 and PI3K by LY294002 resulted in either partial or no inhibition of the target enzyme respectively, similar to the effect of MAPK inhibitor. Taken together, these results suggest that activation of NF-kB by FGF-2 may be an important signaling cascade in the protection of cells from apoptosis by increasing the expression of Bcl2 family genes such as Bcl2-A1 and Bcl-xL.

MATERIALS AND METHODS

Cell culture & reagent

Mouse ATDC5 cells were obtained from RIKEN cell bank (Tsukuba, Japan) and maintained in DMEM/F12 containing 5% FBS (WelGENE, Korea) with antibiotics P/S and 10 mM HEPES (pH 7.4) in 5% CO_2 atmosphere. ATDC5 cells were stained with Crystal Violet (Sigma Cehmical Co., USA) as reported previously (25). FGF-2 was purchased from R&D Systems (Minneapolis, MN, USA). Pyrrolidine dithiocarbamate (PDTC), PD173074, LY294002 and U73122 were from Sigma Chemical Co. (St Louis, MO, USA).

MTT assay

According to the manufacture's protocol, viability of ATDC5 cells was checked by CellTiter 96® Aqueous One Solution Cell proliferation Assay kit (Promega, USA) and microplate reader (Molecular Devices, USA).

RNA isolation and RNase protection assay

Total RNA was isolated from ATDC5 cells using the RNA Blood Mini kit according to the manufacturer's instructions (Qiagen, USA). ATDC5 cells were cultured with or without FGF-2 for 24 hours. Total RNA (5-10 μg) extracted from ATDC5 cells was subjected to an RNase protection assay using the mAPO-2 probe set (BD PharMingen, USA) as specified by the manufacturer. The protected RNAs were resolved on 5% denaturing polyacrylamide gels and exposed to x-ray film.

Real-time polymerase chain reaction

cDNAs were prepared using 5 μg of whole RNA isolated from ATDC5 cells. To quantify the expression level of Bcl2-A1, Bcl2-A1a, Bcl2-A1d, and Bcl-xL, Real-time PCR amplification was performed using the Rotor Gene 3000 PCR machine

(Corbett Research, Mortlak e, Australia) and SYBR Premix EX Taq (TAKARA BIO INC, Japan). The PCR reaction mixture (15 μl) contained 7.5 μl 2X SYBR Premix EX Taq buffer, 0.5 μl of primers (10 pmol/μl), and 1 μl of sample cDNA. The condition for PCR amplification was 40 cycles of 95°C - 2 seconds, 60°C - 15 seconds, 72°C - 20 seconds using gene-specific primers as follows: Bcl2-A1, 5′-CCT GGC TGA GCA CTA CCT TC -3′ and 5′-CCT TCC CCA GTT AAT GAT GC-3′; Bcl2-A1a, 5′-ATA TTT GCC TTT GGG GGT GT-3′ and 5′-AGA AAA GTC AGC CAG CCA GA-3′; Bcl2-A1d, 5′-TCT GAG TAC GAG TTC ATG T-3′ and 5′-CCT TCC CCA GTT AAT GAT GC-3′; Bcl-xL, 5′-TGA AGC AAG CGC TGA GAG-3′ and 5′-GTC ATG CCC GTC AGA AAC-3′; and GAPDH, 5′-GTG AAG GTC GGT GTG AAC G-3′ and 5′-GGT TCA CAC CCA TCA CAA AC-3′.

Immunoblot analysis

Fifty μg of protein extract from each sample was subjected to SDS-PAGE followed by western blotting using antibodies against Bcl2-A1, Bcl-xL, Phospho-IKK, IKK, Phospho-IkB, IkB, Phospho-NF-kB, NF-kB, and tubulin (Santa Cruz Biotechnology, USA).

Immunofluorescence studies

Cells grown on 12-mm cover slips (Fisher Scientific, USA) were incubated with 5 ng FGF-2 for 24 hours, washed with PBS, fixed with 4% formaldehyde for 30 min, then blocked with 10% FBS. Cells were stained with anti-Bcl2-A1 goat antibody (1 : 200 dil.) and anti-Bcl-xL polyclonal antibody (1 : 200 dil.), washed with PBS, and incubated for 30 min with a rhodamine-conjugated secondary antibody. After mounting, cells were analyzed with confocal microscope (Carl Zeiss LSM710, USA).

Small interfering RNA experiments

siRNAs specific for human Bcl2-A1 (5′-CAG AUG ACA GGA CAG AUC UGG GAA A-3′) and Bcl-xL (5′-GCG UAG ACA AGG AGA UGC AGG UAU U-3′) and control siRNA (Scramble Duplex, Bioneer, Daejeon, Korea) were transfected into ATDC5 cells using Lipofectamine RNAiMAX (Invitrogen) (150 pmol/6-well plate, 30 pmol/24-well plate). After 12-48 hours, mRNAs were harvested for RT-PCR analysis. For FACS analysis, ATDC5 cells were transfected with each siRNA and incubated with or without FGF-2 for 36 and 60 hours. Cells were fixed in 75% ethanol and stained with PI for FACS assay (Becton Dickinson, USA).

Electrophoretic mobility shift assay (EMSA)

Nuclear extract was prepared and protein concentration was determined by the Bradford assay (Bio-Rad) to ensure equal amounts of protein in each reaction. Double-stranded DNA probes including NF-kB p65 consensus and mutant sequences were prepared as follows: consensus probe, 5'-AGT TGA GGG GAC TTT CCC AGG C-3'; mutant probe, 5'-AGT TGA GGC GAC TTT CCC AGG C-3'. All binding assays were performed at 30°C for 30 min in 20 μl binding buffer [20 mM HEPES (pH 7.6), 4% (wt/vol) Ficoll type 400, 50 mM KCl, 2 mM EDTA, 2 μg poly (dI-dC)] containing 1 nM ^{32}P-end-labeled probe (20,000-30,000 cpm) and nuclear protein extract. Samples were electrophoresed on 4% polyacrylamide gel in 0.25X Tris-borate-EDTA buffer at 250 V for 1 hour.

Measurement of mitochondrial membrane potential by flow cytometry

ATDC5 cells were incubated with FGF-2 (5 ng/ml) and/or TNF-α (5 ng/ml) before staining with 50 nM 3,3′-diethyloxacarbocyanine iodide (DiOC2 (3); JC-1 dye, Molecular Probes, USA). Cells were analyzed on flow cytometer using 488-nm excitation and 530/30-nm bandpass and 650-nm longpass filters.

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