Dexamethasone promotes osteoblast apoptosis through the Chk2/p53 signaling pathway

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Conflict of interest
None declared

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Abstract

Background. Glucocorticoids (GCs) are widely used to treat inflammatory or autoimmune diseases. However, several studies have reported that the use of GCs can lead to numerous complications, the most serious of which are osteoporosis and osteonecrosis of the femoral head (ONFH). Osteoblast apoptosis has been identified as an important event in the development of GC-induced osteoporosis and ONFH. However, the mechanisms underlying the regulation of these processes have not yet been explored.

Objectives. To observe the effect of dexamethasone (Dex) on the apoptosis of osteoblasts and explore its mechanism, as well as provide a new therapeutic idea for GC-induced osteoporosis and ONFH.

Materials and methods. Cell proliferation and apoptosis of MC3T3-E1 cells after Dex treatment were determined using the CellTiter-Glo® Luminescent Cell Viability Assay kit and Annexin V-FITC/PI Double Staining Apoptosis Detection Kit, respectively. The expression of caspase-3/cleaved caspase-3 and poly(ADP-ribose) polymerase (PARP)/cleaved PARP in MC3T3-E1 cells after Dex treatment was determined with western blotting. The expression of p53 and checkpoint kinase 2 (Chk2) in MC3T3-E1 cells after Dex treatment was analyzed using western blotting and polymerase chain reaction (PCR). The effects of p53 knockdown and Chk2 knockdown on Dex-induced apoptosis of MC3T3-E1 cells were also characterized.

Results. Dexamethasone remarkably inhibited cell growth and induced the apoptosis of MC3T3-E1 cells. We also observed that Dex induced osteoblast apoptosis by promoting p53 expression. The regulatory effect of Dex on p53 expression is mediated by the upregulation of Chk2, which interacted with p53 and inhibited p53 degradation. The knockdown of p53 alleviated Dex-induced MC3T3-E1 cell apoptosis by decreasing the expression of cleaved caspase-3 and cleaved PARP.

Conclusions. We demonstrated that Dex increased Chk2 protein expression, which stabilized the protein expression of p53, and in turn promoted osteoblast apoptosis.

Key words: dexamethasone, osteoblast apoptosis, Chk2/p53, glucocorticoid (GC)-induced osteoporosis, ONFH

Background

Glucocorticoids (GCs) are among the most commonly prescribed drugs. As they have anti-inflammatory and immunosuppressive properties, GCs are primarily used to treat inflammatory or autoimmune diseases, such as systemic lupus erythematosus and nephrotic syndrome.[1,2] However, recent studies have revealed that the use of GCs can cause many undesirable complications. Among these, osteoporosis and osteonecrosis of the femoral head (ONFH) are the most serious.[3–5] There is evidence that osteoblast apoptosis is an important event in the development of osteoporosis and ONFH induced by GCs.[6]

Dexamethasone (Dex) is a synthetic GC and its functions include inhibiting collagen and fibronectin synthesis[7] and activating collagenase synthesis.[8] Although Dex has been reported to induce apoptosis of osteoblasts and exert a modulatory effect on osteoblast proliferation,[9,10] the regulation of these mechanisms by Dex is yet to be characterized.

Checkpoint kinase 2 (Chk2) is an important regulator that can rapidly phosphorylate downstream effectors and lead to DNA damage.[11] When Chk2 is activated, it leads to alternative cell responses, such as programmed cell death.[12] The Chk2/p53 pathway can induce cell cycle arrest in DNA damage responses (DDRs) and activate p53-related apoptosis pathways.[13] It has been reported that Dex can cause cell apoptosis by upregulating the expression of p53 in MC3T3-E1 cells.[14] However, the role and mechanisms of the Chk2/p53 pathway in osteoblasts have not been determined. Accordingly, we aimed to characterize the molecular mechanisms underlying Dex promotion of osteoblast cell apoptosis.

Objectives

The objective of this study was to observe the effect of Dex on the apoptosis of osteoblasts and explore the underlying mechanisms. This could reveal a new therapeutic potential for GC-induced osteoporosis and ONFH.

Materials and methods

Reagents, plasmids and antibodies

The SuperScript™ First-Strand Synthesis System and TRIzol Reagent were acquired from Invitrogen (Grand Island, USA). The plasmids of shp53 and shChk2 were obtained from Open Biosystems (Thermo Fisher Scientific, Pittsburgh, USA). Antibodies against caspase-3, cleaved caspase-3, poly(ADP-ribose) polymerase (PARP), cleaved PARP, p53, p-p53 Ser15, p-p53 Ser20, ataxia telangiectasia mutated kinase and Rad3-related kinase (ATR), p-ATR Ser428, murine double minute 2 (MDM2), p-MDM2 Ser166, p-Chk2 Thr68, p-Chk1 Ser345, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin were purchased from Cell Signaling Technology (Danvers, USA). Dexamethasone was obtained from Sigma-Aldrich (St. Louis, USA). Cycloheximide (CHX) and MG132 were acquired from Calbiochem (San Diego, USA).

Cell culture

Osteoblast cells (MC3T3-E1) were obtained from the Institute of Biochemistry and Cell Biology (IBCB; Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) high glucose (Gibco, Waltham, USA) was used to maintain cells. It was synchronized by incubation in medium with extra 0.1% fetal bovine serum (FBS; NovaTech, Grand Island, USA) overnight, and cultured in medium with extra 10% FBS and penicillin-streptomycin solution (100 U/mL penicillin and 100 μg/mL streptomycin). The culture was maintained in a humidified incubator atmosphere with 5% CO_2 at 37°C.

Transfection

In this study, we used the PolyJet™ In Vitro DNA Transfection Reagent (SignaGen Laboratories, Rockville, USA) to transfect cells. In order to obtain stable transfected cells, the cells were cultured with hygromycin B, G418 or puromycin according to the transfected granules with different antibiotic resistance properties. Transfected cells were selected with hygromycin B, G418 or puromycin for 3–4 weeks. Before being used for further experiments, cells were cultured for at least 2 passages.

Cell proliferation assay

Adenosine triphosphate (ATP)ase analysis was used to evaluate the effect of Dex on viability of MC3T3-E1 cells. Cells were cultured in 96-well plates with a density of 3×10^3 cells per well and subsequently treated with 1 μM Dex for different durations (1–4 days). Treated cells were collected and analyzed with ATP Colorimetric/Fluorometric Assay Kit (Sigma-Aldrich), following the manufacturer's instructions.

Determination of cell apoptosis

The MC3T3-E1 cells were incubated to 70–80% confluence in 6-well plates. Before the Dex treatment, the culture medium was shifted to 0.1% FBS medium for 24 h. The cells were then treated with the designated concentration of Dex. After the treatment that lasted for 24 h, the cells were washed with phosphate-buffered saline (PBS) 3 times. Then, 100 μL of cells were incubated with equal volume of FITC-Annexin V/PI Assay Kit solution (Biosea Biotechnology, Beijing, China) in the dark for 30 min

at room temperature. The apoptosis rate was recorded using a flow cytometer (Bender MedSystems, Burlingame, USA).

Protein degradation experiments

The MC3T3-E1 cells were pretreated with the proteasome inhibitor MG132 at a concentration of 20 μM for 24 h. The MG132 was then removed and 100 μg/mL of synthetic inhibitor CHX were added to the culture medium – alone or in combination with 1 μM Dex in different time intervals (2 h, 4 h or 6 h). The levels of remaining p53 and GAPDH proteins were detected by means of western blot analysis.

Western blot analysis

Cells (1×10^6) were inoculated on 6-well plates and treated by the method described previously. Then, they were extracted with cell lysis buffer. For each sample, 60–80 μg of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The membrane was then incubated with primary antibodies overnight, followed by incubation with the alkaline phosphatase (AP)-conjugated secondary antibody. The ImageQuant™ TL software v. 8.1 (Cytiva, Marlborough, USA) was used to detect the density of the bands. The results shown in this study represent 3 independent experiments.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) for miRNA assay

The RNeasy Mini Kit (Qiagen, Valencia, USA) was used to extract total miRNAs. The MC3T3-E1 cells (1×10^6) were inoculated on 6-well plates and treated with 1 μM Dex for different time periods (1–3 h). For the reverse transcription, the amount of total RNA used was 1 μg. The expression of *p53* was amplified using the miScript PCR Kit (Qiagen) and analyzed using the 7900HT Fast Real-time PCR system (Applied Biosystems, Carlsbad, USA). The primers were: human *p53* (forward: 5'-ACCTATGGAAACTACTTCCTGAAA-3', reverse: 5'-CTGGCATTCTGGGAGCTTCA-3').

Statistical analyses

The experimental data were presented as mean ± standard deviation (M ±SD) and analyzed with IBM SPSS v. 19.0 statistical software (IBM Corp., Armonk, USA). The normality of the data was analyzed using the Shapiro–Wilk test, and the value of $p > 0.05$ indicated that the data were normally distributed. The Levene's test was used to analyze the homogeneity of variance, and the value of $p > 0.05$ indicated that the data conformed to homogeneity of variance. The one-way analysis of variance (ANOVA) test for repeated measures, followed by the post hoc least significant difference (LSD) test were used for overall comparisons between groups across multiple time points. The value of $p < 0.05$ was considered statistically significant.

Results

Dex significantly inhibited the proliferation of MC3T3-E1 cells and induced cell apoptosis

We used the ATPase analysis to evaluate the effect of Dex on the viability of MC3T3-E1 cells. Cells were cultured in the Dex dose range (1 μM). The Shapiro–Wilk test was used to analyze the normality of the data. As shown in Table 1, the data were normally distributed ($p > 0.05$). The homogeneity of variance test indicated that the data conform to homogeneity of variance (Table 2, $p > 0.05$). As shown in Fig. 1A, cell apoptosis had not yet started over the first 2 days and there was no statistically significant difference between the treatment groups (day 1: $F = 3.654$, $p = 0.196$; day 2: $F = 2.476$; $p = 0.256$). In contrast, cell viability was significantly reduced in Dex-treated cells on days 3 and 4 (day 3: $F = 73.491$, $p = 0.013$; day 4: $F = 123.085$; $p = 0.008$). Specific data points are shown in Table 3. We then used FITC-Annexin V/PI staining to detect the effect of Dex on apoptosis in MC3T3-E1 cells. As shown in Fig. 1B,

Table 1. Shapiro–Wilk test results of the effect of dexamethasone (Dex) on the viability of MC3T3-E1 cells (M ±SD)

Test	n	Data	Statistics	p-value
Medium control day 1	3	3.768 ±1.207	0.966	0.648
Medium control day 2	3	6.228 ±1.609	0.868	0.289
Medium control day 3	3	14.417 ±0.560	0.962	0.625
Medium control day 4	3	21.739 ±2.030	0.948	0.560
Dex 1 μM day 1	3	2.472 ±0.640	0.858	0.261
Dex 1 μM day 2	3	3.938 ±1.518	0.977	0.708
Dex 1 μM day 3	3	7.237 ±1.002	0.997	0.891
Dex 1 μM day 4	3	9.329 ±0.983	0.998	0.909

M ±SD – mean ± standard deviation.

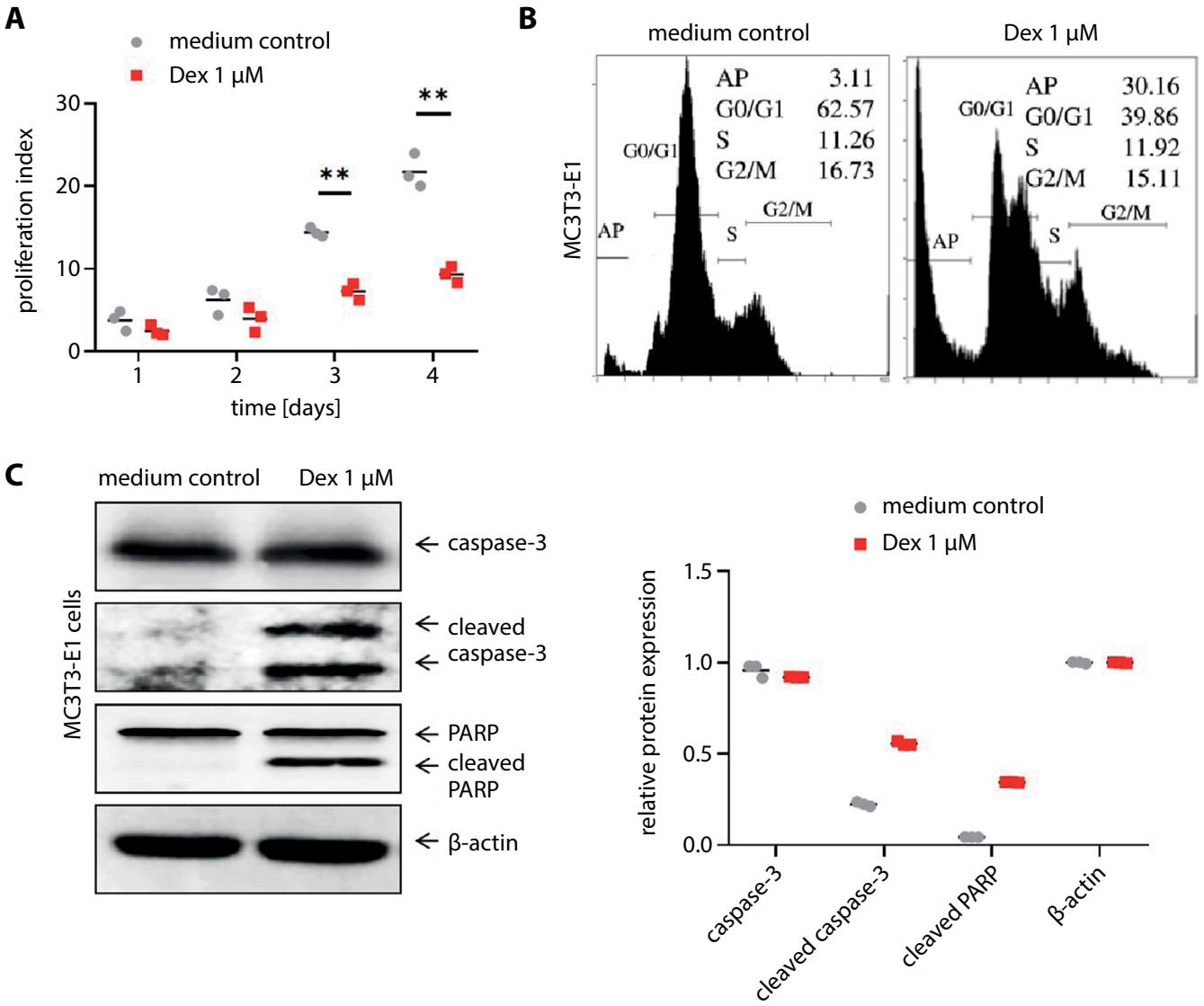

Fig. 1. Dexamethasone (Dex) significantly inhibited the proliferation of MC3T3-E1 cells and induced cell apoptosis. A. Cell viability was significantly reduced after Dex treatment (** $p < 0.01$ on day 3 and day 4); B. The cell apoptosis analysis on MC3T3-E1 cells after Dex treatment; C. Western blot analysis on the expression of caspase-3/cleaved caspase-3 and poly (ADP-ribose) polymerase (PARP)/cleaved PARP in MC3T3-E1 cells after Dex treatment

Table 2. Levene's test of the homogeneity of dexamethasone (Dex) with the viability of MC3T3-E1 cells

Test	Day 1	Day 2	Day 3	Day 4
F	1.268	0.064	0.662	1.934
p-value	0.323	0.813	0.461	0.237

Table 3. Comparison of cell viability at each time point of dexamethasone (Dex) administration with the viability of MC3T3-E1 cells

Test	Day 1	Day 2	Day 3	Day 4
F	3.654	2.476	73.491	123.085
p-value	0.196	0.256	0.013	0.008

Treatment factor main effect: $F = 112.41$, $p < 0.01$; time factor main effect: $F = 115.99$, $p < 0.01$; interaction between the 2 factors: $F = 23.76$, $p < 0.01$.

we found that the apoptosis rate of MC3T3-E1 cells significantly increased after 24 h of Dex treatment. Considering that PARP cleavage and caspase activation are typically associated with apoptosis, we used western blotting in order to detect the expression of these proteins in Dex-treated MC3T3-E1 cells. The results suggested that the cleavage of cleaved PARP and cleaved caspase-3 was increased by Dex treatment in MC3T3-E1 cells (Fig. 1C). Collectively, our results demonstrate that Dex can significantly inhibit the proliferation of MC3T3-E1 cells and induce the cell apoptosis.

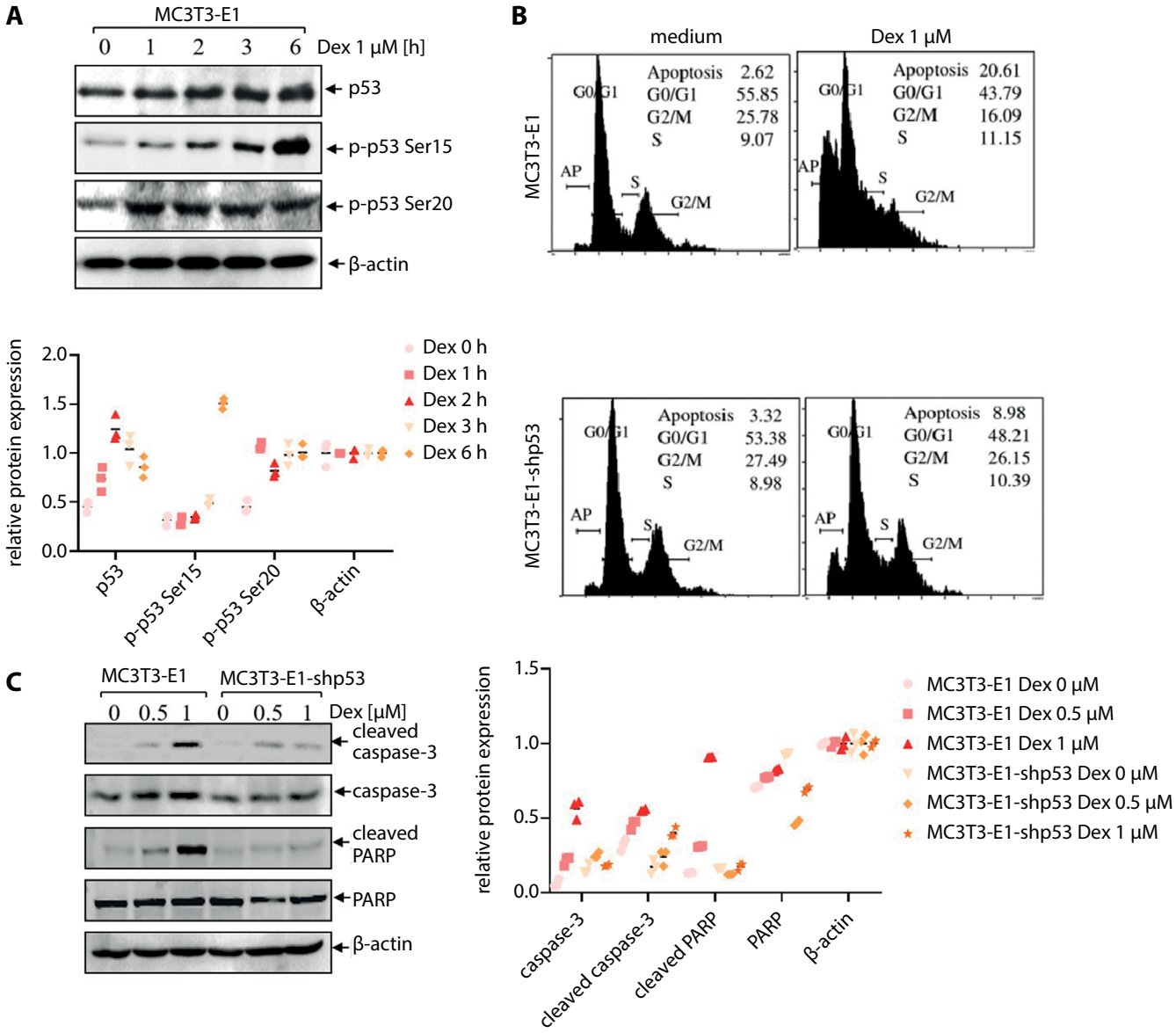

Fig. 2. The phosphorylation of p53 protein was important for the induction of apoptosis following dexamethasone (Dex) treatment. A. The expression of p53 protein phosphorylated at Ser15, Ser20 and total p53 protein in Dex-treated MC3T3-E1 cells; B. Cell apoptosis induced by Dex observed using flow cytometric analysis; C. Western blot analysis indicated that Dex-induced cleavage of poly (ADP-ribose) polymerase (PARP) and caspase-3 were blocked by p53 protein knockdown

Phosphorylation of p53 was crucial for apoptosis induction after Dex treatment

It has been reported that p53 protein is associated with caspase-dependent apoptosis.[15] In order to elucidate the molecular mechanisms underlying the apoptotic effect of Dex, western blotting was performed to determine whether p53 was involved in the cell apoptosis induced by Dex. As presented in Fig. 2A, the expression of total p53 protein and phosphorylated status of p53 protein at Ser15 and Ser20 were determined in MC3T3-E1 cells after Dex treatment. The phosphorylation level of p53 protein at Ser20 was significantly upregulated 1 h after the exposure. Similarly, the phosphorylation level of p53 protein at Ser15 slightly increased in the early treatment period (≤3 h) and significantly increased 6 h after treatment. The expression of total p53 protein showed a stable increase after Dex treatment and reached a peak within 2–6 h following the treatment. As phosphorylation at Ser20 usually contributes to the stabilization of p53 protein and results in the phosphorylation at Ser15,[10] we presumed that the Dex-induced p53 activation in MC3T3-E1 cells was also dependent on phosphorylation of Ser20.

To further confirm whether Dex-induced apoptosis requires p53 activation, we used shp53 MC3T3-E1 cells. The flow cytometry analysis demonstrated that the apoptosis induction rate of shp53 MC3T3-E1 cells induced by Dex was much lower than that of wild-type MC3T3-E1 cells (Fig. 2B). Western blot results indicated that the knockdown of p53 could block the cleavage

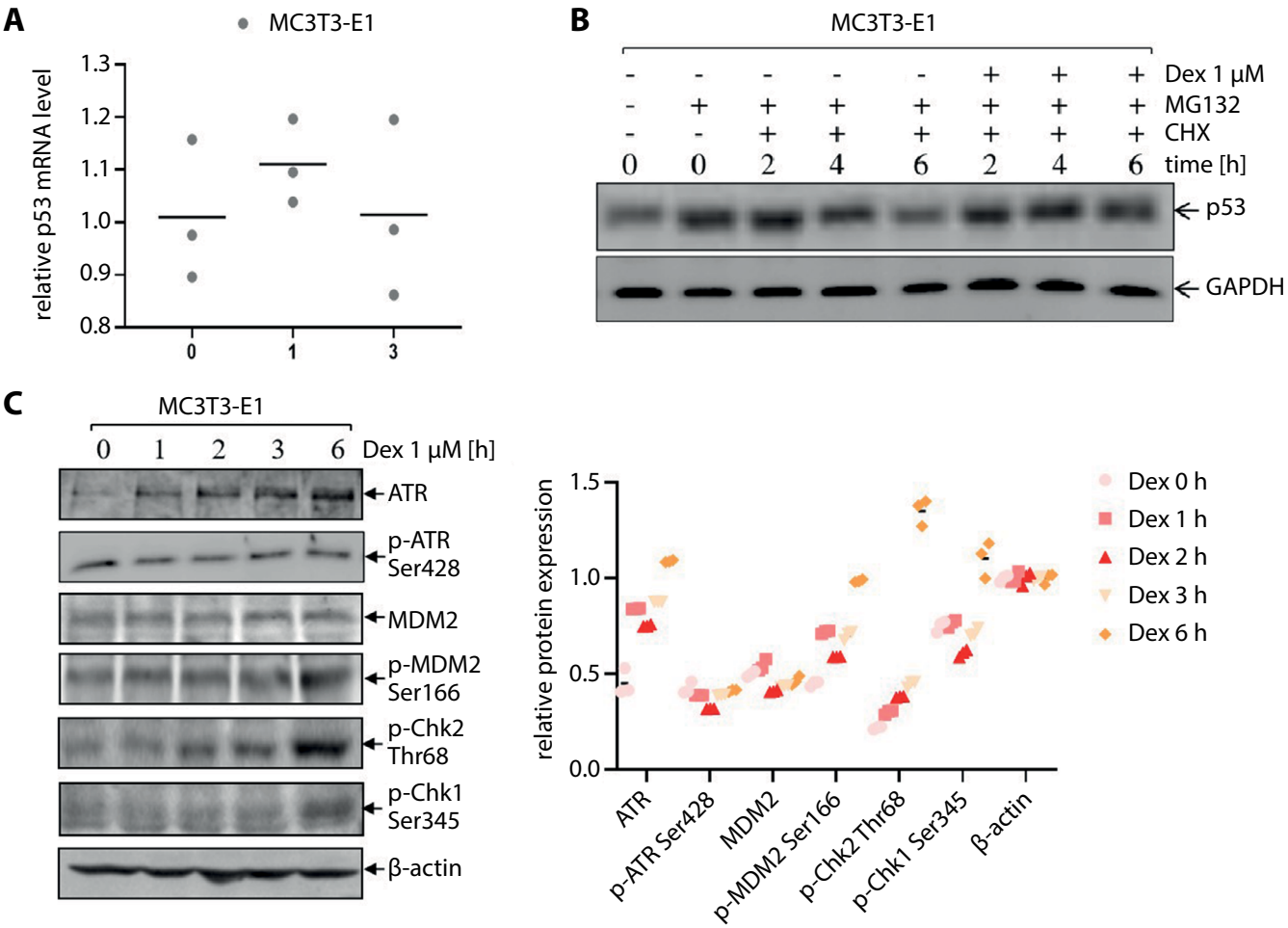

Fig. 3. Dexamethasone (Dex) induced caspase-dependent apoptosis by promoting p53 protein degradation. A. Quantitative polymerase chain reaction (qPCR) analysis of Dex-treated MC3T3-E1 cells; B. MC3T3-E1 cells were pretreated with MG132. Western blot analysis on the dynamics of p53 protein degradation induced by Dex; C. Western blot analysis on phosphorylation of ataxia telangiectasia mutated kinase and Rad3-related kinase (ATR) protein at Ser428, murine double minute 2 (MDM2) protein at Ser166, checkpoint kinase 2 (Chk2) protein at Ser345, and Chk2 protein at Thr68 in Dex-treated MC3T3-E1 cells

CHX – cycloheximide; GAPDH – glyceraldehyde-3-phosphate dehydrogenase.

of PARP and caspase-3 induced by Dex (Fig. 2C). Taken together, these results suggest that the activation of p53 protein is essential for cell apoptosis in MC3T3-E1 cells induced by Dex treatment.

Dex induced caspase-dependent apoptosis through promoting p53 protein degradation

In order to investigate the mechanism of Dex upregulation of p53 protein, we characterized the effect of Dex on the expression of *p53* mRNA level in MC3T3-E1 cells. The results of quantitative PCR have shown that Dex had no effect on the expression of *p53* mRNA level after 3 h of treatment (Fig. 3A). Therefore, we inferred that Dex might regulate the induction of p53 protein at the protein level instead.

In order to elucidate whether Dex was able to prevent p53 protein degradation, we pretreated MC3T3-E1 cells with the proteasome inhibitor MG132. The MG132 was subsequently removed and the synthetic inhibitor CHX was added into the medium, alone or in combination with Dex, for different time periods.[17] This experiment could provide a basis for further research on the effects of Dex on the degradation dynamics of p53 protein. After the cells were treated with both Dex and CHX for 4 h and 6 h, the expression level of p53 protein in the cells was much higher than in the cells treated with CHX alone (Fig. 3B). These results suggest that Dex was contributing to the accumulation of p53 by inhibiting its proteasome-mediated degradation.

Chk2 protein regulates p53 protein degradation during Dex treatment

The degradation of p53 protein can be regulated by many factors, such as MDM2, Chk1/2 and ATR.[18] For instance, studies have shown that the post-translational phosphorylation of MDM2 protein at Ser166 contributes to the degradation of p53 protein.[19,20] Accordingly,

we examined the phosphorylation of MDM2 at Ser166 in Dex-treated MC3T3-E1 cells. The expression of total MDM2 protein was not affected by Dex, while the phosphorylation level at Ser166 was slightly upregulated after Dex treatment at 6 h (Fig. 3C). These results suggested that Dex did not inhibit the phosphorylation of the MDM2 protein at Ser166. Therefore, it could not be explained why p53 protein accumulated in MC3T3-E1 cells after Dex treatment.

Several studies have shown that Chk1/2 can also affect the stabilization and activation of p53 protein by inducing its phosphorylation.[21] As phosphorylation of Chk1 protein at Ser345 or Chk2 protein at Thr68 is necessary for p53 protein activation,[22,23] we evaluated the phosphorylation state of both sites in Chk1 and Chk2 after Dex treatment. Western blot results show that Chk2 protein was phosphorylated at Thr68 after 2 h of Dex treatment and reached a peak after 6 h of treatment, while the phosphorylation level of Chk1 protein at Ser345 increased slightly after 6 h of Dex treatment (Fig. 3C). As p53 is phosphorylated at an early stage, we eliminated the possibility that Chk1 protein is directly involved in inducing p53 protein and hypothesized that Chk2 protein may have some functions in p53 protein stabilization and reactivation instead.

Chk2 protein led to p53-mediated cell apoptosis during Dex treatment

To illuminate the mechanisms underlying Chk2 promotion of p53 protein expression, MC3T3-E1 ShChk2 cells were used to analyze phosphorylation and expression levels of p53 protein after Dex treatment. Western blot analysis indicated that phosphorylated Chk2 protein was depleted in MC3T3-E1 ShChk2 cells compared to MC3T3-E1 cells (Fig. 4A). Importantly, the expression of p53 protein was inhibited in MC3T3-E1 ShChk2 cells induced by Dex and when Chk2 was knocked down, the phosphorylation of p53 protein at Ser15 and Ser20 was nearly eliminated. As the phosphorylation of p53 protein at Ser20 typically contributes to the stability of p53 protein, which leads to the phosphorylation of p53 protein at Ser15,[10] we predicted that Chk2 could regulate the phosphorylation of p53 protein at Ser15 and Ser20 and mediate Dex-induced p53 protein stabilization and accumulation. In order to investigate whether the accumulation of p53 mediated by Chk2 was the result of preventing the degradation of p53 protein, MC3T3-E1 ShChk2 cells were treated with MG132 and CHX. The results revealed that there was no effect of Dex on the inhibition of p53 protein degradation in MC3T3-E1 ShChk2 cells under the same conditions (Fig. 4B). Further studies revealed that Chk2 protein enhanced the stability of Dex-induced p53 protein expression. Similarly, the loss of Chk2 led to apoptosis (Fig. 4C) and caspase-3 cleavage (Fig. 4D). These results indicate that the expression of Chk2 protein was essential for the phosphorylation of p53 protein, which in turn increased p53 protein stabilization and accumulation, and thus resulted in cell apoptosis after Dex treatment.

Discussion

Dexamethasone has been reported to induce apoptosis of osteoblasts and exert a modulatory effect on their proliferation.[24] However, the regulation of these mechanisms by Dex has not been explored yet. Therefore, this study revealed a novel biological role of Dex in the induction of cell apoptosis and inhibition of colony formation in MC3T3-E1 cells. The mechanism studies demonstrated that Dex-mediated apoptosis was via the Chk2/p53 pathway. This finding suggests that Chk2/p53 might also serve as a potential intervention in ONFH treatment.

Dexamethasone can upregulate the expression of p53 protein in MC3T3-E1 cells and cause cell apoptosis.[25] However, the role and mechanism of the Chk2/p53 pathway on osteoblasts is yet to be elucidated. Interestingly, our results show that Dex-induced cell apoptosis in MC3T3-E1 cells was mediated by the accumulation of p53 protein. In particular, Dex could promote p53 protein stabilization by inducing Chk2 phosphorylation. Furthermore, our results strongly indicated that Dex can regulate p53 protein by preventing its degradation.

The degradation of p53 protein can be caused by a variety of factors, such as the activation of MDM2, Chk1, Chk2, and ATR.[26–28] Our study demonstrated that MDM2 and Chk1 were not direct regulators of Dex upregulation of p53 protein in MC3T3-E1 cells, but that the activation of Chk2 occurred relatively early. This suggested that Chk2 may involved in the induction of p53 protein by Dex treatment.

Limitations

Our research indicated the molecular mechanisms underlying Dex promotion of osteoblast cell apoptosis. However, there are 2 limitations of this study. First, we did not evaluate other effects of GCs (including Dex) in the treatment of inflammatory or autoimmune diseases. As a side effect of their clinical applications, GCs can cause osteonecrosis and ONFH.[29–31] Previous research had shown that the damaging effects of Dex on osteoblasts and ONFH are due to its role in inhibiting cell proliferation and promoting cell apoptosis.[32,33] Therefore, we only considered the effect of Dex on inhibiting cell proliferation and promoting cell apoptosis. Second, in this study, we demonstrated that Dex can inhibit MC3T3-E1 proliferation and induce apoptosis, but we did not evaluate other pathways in apoptosis induced by Dex. In our previous study, we found that Dex can upregulate the expression of p53 protein in MC3T3-E1 cells, thus causing cell apoptosis.[14] Various studies have shown that the Chk2/p53 pathway can induce growth inhibition and apoptosis.[34,35]

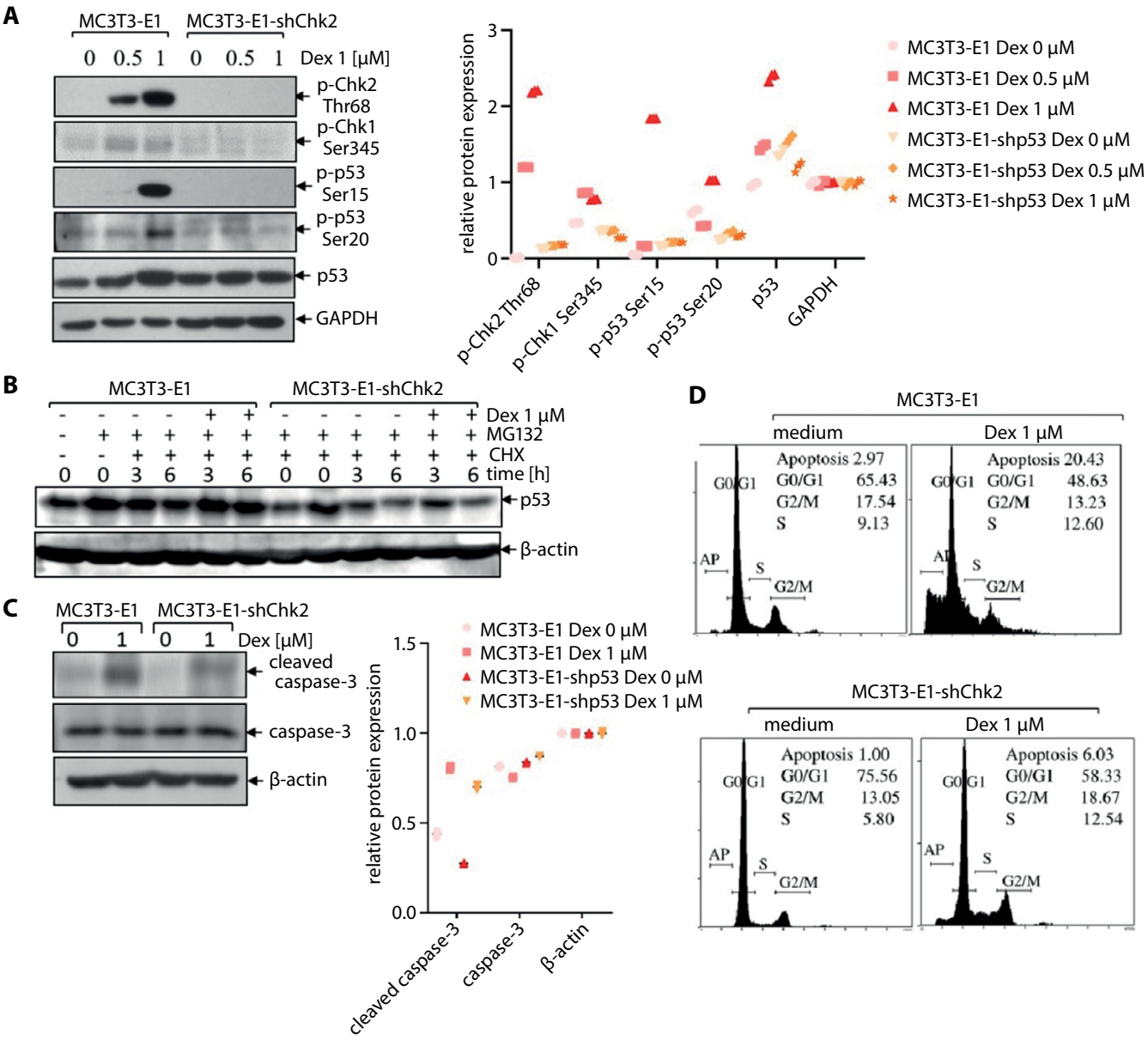

Fig. 4. Checkpoint kinase 2 (Chk2) led to p53-mediated apoptosis during dexamethasone (Dex) treatment. A. Phosphorylation and expression of p53 protein after Dex treatment of MC3T3-E1 ShChk2 cells; B. The effect of Dex inhibition of p53 protein degradation in MC3T3-E1 cells and MC3T3-E1 ShChk2 cells; C. Chk2 mediated Dex-induced p53 protein expression; D. Flow cytometry analysis on the apoptosis-inducing effects on MC3T3-E1 cells and MC3T3-E1 ShChk2 cells after Dex treatment

CHX – cycloheximide; GAPDH – glyceraldehyde-3-phosphate dehydrogenase.

In line with this, our results show that Dex can activate p53-mediated cell apoptosis in MC3T3-E1 cells through the Chk2/p53 signaling pathway. The practical implications of these results need to be further analyzed, particularly in clinical applications.

Conclusions

In summary, our studies demonstrated that Dex could promote cell apoptosis in MC3T3-E1 cells. Our investigation of apoptosis induction showed that Dex mediated the phosphorylation and activation of Chk2. Activated Chk2 leads to the accumulation of p53 protein through the Chk2/p53 signaling pathway, which in turn results in cell apoptosis. Our research can provide a new therapeutic avenue for GC-induced osteoporosis and ONFH.

The flowchart of this study was shown in Fig. 5.

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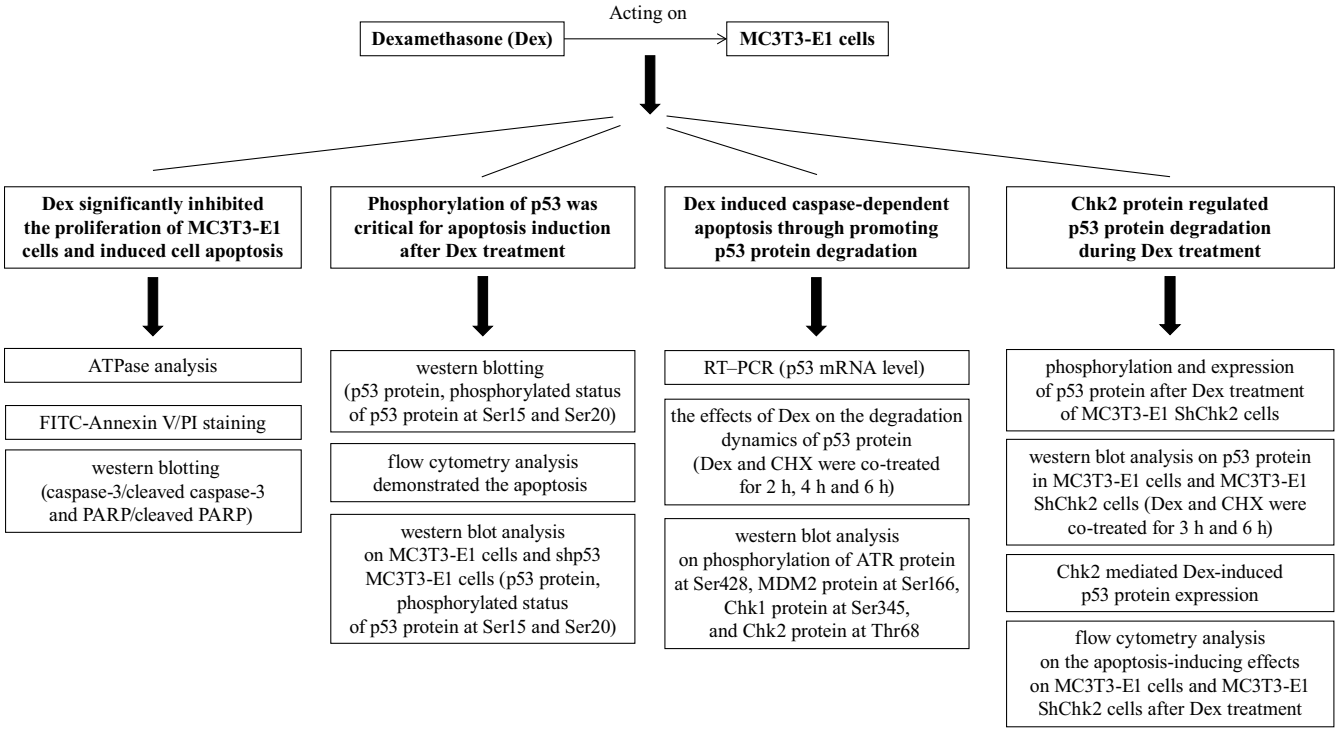

Fig. 5. Flowchart of the study

ATP – adenosine triphosphate; PARP – poly(ADP-ribose) polymerase; RT-PCR – reverse transcription-polymerase chain reaction; CHX – cycloheximide; ATR ataxia telangiectasia mutated kinase and Rad3-related kinase; MDM2 – murine double minute 2.

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