Quantitative proteomic analysis of dexamethasone-induced effects on osteoblast differentiation, proliferation, and apoptosis in MC3T3-E1 cells using SILAC

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Abstract

Summary The impairment of osteoblast differentiation is one cause of the glucocorticoid-induced osteoporosis (GCOP). The quantitative proteomic analysis of the dexamethasone (DEX)-induced effects of osteoblast differentiation, proliferation, and apoptosis using stable-isotope labeling by amino acids in cell culture (SILAC) demonstrated drastic changes of some key proteins in MC3T3-E1 cells.

Introduction The impairment of osteoblast differentiation is one of the main explanations of GCOP. SILAC enables accurate quantitative proteomic analysis of protein changes in cells to explore the underlying mechanism of GCOP.

Methods Osteoprogenitor MC3T3-E1 cells were treated with or without 10^{-6} M DEX for 7 days, and the differentiation ability, proliferation, and apoptosis of the cells were measured. The protein level changes were analyzed using SILAC and liquid chromatography-coupled tandem mass spectrometry.

Results In this study, 10^{-6} M DEX inhibited both osteoblast differentiation and proliferation but induced apoptosis in osteoprogenitor MC3T3-E1 cells on day 7. We found that 10^{-6} M DEX increased the levels of tubulins (TUBA1A, TUBB2B, and TUBB5), IQGAP1, S100 proteins (S100A11, S100A6, S100A4, and S100A10), myosin proteins (MYH9 and MYH11), and apoptosis and stress proteins, while inhibited the protein levels of ATP synthases (ATP5O, ATP5H, ATP5A1, and ATP5F1), G3BP-1, and Ras-related proteins (Rab-1A, Rab-2A, and Rab-7) in MC3T3-E1 cells.

Conclusions Several members of the ATP synthases, myosin proteins, small GTPase superfamily, and S100 proteins may participate in functional inhibition of osteoblast progenitor cells by GCs. Such protein expression changes may be of pathological significance in coping with GCOP.

Keywords Apoptosis · Dexamethasone · Osteoblast differentiation · Proliferation · Proteomics · SILAC

Abbreviations

ACTA Actin, alpha skeletal muscle
ACTB Actin, cytoplasmic 1
ALP Alkaline phosphatase
ANXA1 Annexin A1
ANXA8 Annexin A8
ATP5A1 ATP synthase subunit alpha, mitochondrial precursor
ATP5F1 ATP synthase B chain, mitochondrial precursor
ATP5H  ATP synthase D chain, mitochondrial  
ATP5O  ATP synthase O subunit, mitochondrial precursor  
BAX  Apoptosis regulator BAX  
DEX  Dexamethasone  
G3BP1  Ras GTPase-activating protein-binding protein 1  
GAPDH  Glyceraldehyde-3-phosphate dehydrogenase  
GCOP  GC-induced osteoporosis  
GC  Glucocorticoid  
HSPA4  Heat shock 70 kDa protein 4  
HSP90AA1  Heat shock protein HSP 90-alpha  
IQGAP1  Ras GTPase-activating-like protein 1  
INTS3  Integrator complex subunit 3  
RAB  Ras-related protein  
SILAC  Stable-isotope labeling by amino acids in cell culture  
TUBA1A  Tubulin alpha-1 chain  
TUBB2B  Tubulin beta-2B chain  
TUBB5  Tubulin beta-5 chain  
LC-MS/MS  Liquid chromatography-coupled tandem mass spectrometry  
MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
MYH9  Myosin 9  
MYBBP1A  Myb-binding protein 1A  
MYH11  Isoform 1 of myosin 11  
PDCD6IP  Programmed cell death 6-interacting protein  
PDCD6  Programmed cell death 6  
PIGOK  Protein Interrogation of Gene Ontology and KEGG databases  
PTRF  Polymerase I and transcript release factor  
PPIB  Peptidylprolyl isomerase B  
VCP  Transitional endoplasmic reticulum ATPase

Introduction

Glucocorticoids (GCs) are naturally produced steroid hormones or synthetic compounds that are widely used to treat the inflammation and autoimmune disorders. The bone loss and osteoporosis are major side effects if the GCs are administrated at high doses and in long term. The impairment of osteoblast differentiation and bone formation is one of the main explanations of GC-induced osteoporosis (GCOP) [1, 2]. GCs have been shown to inhibit osteoblast differentiation through downregulation of some key proteins, such as bone morphogenetic protein 2, growth hormone, insulin-like growth factors I and II, alkaline phosphatase (ALP), type I collagen, osteocalcin, and osteopontin, or through upregulation of certain proteins such as MKP-1, Notch-1, collagenases 1, and collagenase 3 [3]. In addition, GCs may also inhibit osteoblast differentiation by opposing Wnt-β-catenin signaling pathway, a complex network of proteins well known for their roles in promoting osteoblastogenesis [4, 5]. Other signaling pathways involving the impacts of GCs on osteoblast differentiation may be Krox 20/EGR2, MPK-1, Notch, and TGF-b-Smad pathway [6–9]. However, along with their effects on osteoblast differentiation, GCs may also affect cell metabolism, proliferation, apoptosis, and other biological functions, which may in turn affect the osteoblastogenesis.

Stable-isotope labeling by amino acids in cell culture (SILAC) enables metabolic incorporation of isotope mass tags into proteins [10]. The cells representing two biological conditions are grown in normal medium (light medium) and isotope amino acid labeled medium (heavy medium), respectively. After grown for six cell divisions in labeled medium to reach 100% of the incorporation of the isotope amino acid, the cells are processed for further treatment, followed by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) analysis. Therefore, the relative intensity ratio of the two pairs of “light” and “heavy” peaks in the peptide mass spectrum can reflect the protein quantitative expression levels under different conditions [10].

This method allows us to apply SILAC for investigation of the underlying mechanism of GCOP. In this study, a high dose of 10⁻⁶ M dexamethasone (DEX) was used to inhibit the biological function of osteoblast progenitor MC3T3-E1 cells, and quantitative proteomic analysis based on SILAC and LC-MS/MS was performed to explore the protein level changes in the process of DEX-induced inhibition in MC3T3-E1 cells.

Methods

Media composition

The SILAC™ protein ID and quantitation media kit (DMEM Flex) was purchased from Invitrogen Corporation (Carlsbad, CA, USA). The isotope l-lysine labeled heavy medium and normal light medium were reconstituted according to the manufacturer’s instructions. The heavy medium contained 100 mg/ml heavy [U-13C6] L-lysine in DMEM basal medium, while the light medium had 100 mg/ml light [U-12C6] L-lysine. Additionally, both heavy and light media were supplemented with 4,500 mg d-glucose, 100 mM L-glutamine, 10% dialyzed fetal bovine serum, 100 mg/ml light L-arginine, and 1% penicillin/streptomycin.

Cell proliferation assay

The MC3T3-E1 cells were separated into 10⁻⁶ M DEX treatment (DEX) group and control (CON) group and...
seeded at a 96-well plate at the concentration of \(1.0 \times 10^4\) cells/cm². The cells in DEX group were grown in a normal medium containing \(10^{-6}\) M DEX, while the cells in CON group were raised in a normal medium without DEX. After 7-day culture in 5% CO₂ at 37°C, the cell proliferation rate was measured by the reduction rate of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) into formazan. In brief, the cells were incubated with 0.5 mg/ml MTT for 4 h, and the formazan was then dissolved with 10% SDS in 0.01 M HCL at 37°C overnight. Absorbance at 590 nm was determined using an MCC 340 multi-scan microplate reader (Thermoelectron, MA, USA).

Apoptosis assay

The MC3T3-E1 cells were separated into DEX and CON group and seeded at a 6-well plate at the concentration of \(1.0 \times 10^4\) cells/cm², and cultured in normal media with or without \(10^{-6}\) M DEX for 1, 4, 7, and 14 days. Microscopic detection of apoptosis was carried out on adherent cells after treatment with 10 μg/ml Hoechst 33342 for 15 min at 37°C. Slides were visualized under ultraviolet filters. Cells with apoptotic nuclei were counted in at least five different fields and expressed as percentage of total cells counted.

ALP activity assay

The MC3T3-E1 cells were separated into DEX and CON groups, seeded in 6-well plates at the concentration of \(1.0 \times 10^4\) cells/cm², and cultured in normal media with or without \(10^{-6}\) M DEX for 1, 4, 7, and 14 days before harvesting. The ALP activities in cell lysates were quantitatively analyzed using a SensoLyte pNPP ALP Assay Kit (AnaSpec, San Jose, CA, USA) following the manufacturer’s instruction. The values were normalized to the protein concentration measured by a Bio-Rad Protein Assay reagent (BioRad, Hercules, CA, USA) with bovine serum albumin (BSA) used as the standard.

SILAC in MC3T3-E1 cells

MC3T3-E1 cells were expanded for six cell divisions in the heavy medium or light medium in 5% CO₂ at 37°C to reach more than 97% of incorporation. Then, the cells were seeded in 75-cm² plastic flasks (Corning, New York, NY, USA) at the concentration of \(1.0 \times 10^5\) cells/cm². The MC3T3-E1 cells in DEX group were cultured in light medium and contained \(10^{-6}\) M DEX for 7 days, while the cells in CON group were grown in heavy medium for 7 days before harvesting.

Cell lysis and protein extraction

The MC3T3-E1 cells were then trypsinized and washed with phosphate buffer solution three times. Equal number of cells (\(2.0 \times 10^7\)) from both DEX and CON groups were mixed and homogenized in NP40 Cell Lysis Buffer (BioSource™, Camarillo, CA, USA) complemented with 0.01 m dithiothreitol and protease inhibitor. The homogenates were centrifuged at 14, 000 rpm for 25 min at 4°C to collect the supernatants.

Western blotting analysis

Twenty-milligram protein samples were separated by a ten-well Novex® 4–12% Tris–glycine gel (Invitrogen, Carlsbad, CA, USA) and transferred to a polyvinylidene difluoride membrane. After blocked using 1% BSA, each membrane was incubated for 24 h at 4°C with one of the following antibodies: rabbit polyclonal S100A4 antibody (Abacam, Cambridge, UK), rabbit polyclonal Rab7-117 antibody (Abacam, Cambridge, UK), rabbit polyclonal ATP5O antibody (Abacam, Cambridge, UK), and rabbit polyclonal β-actin antibody (Santa Cruz, CA, USA). The membranes were then incubated with HRP-conjugated anti-rabbit IgG (Santa Cruz, CA, USA), and the proteins on the membranes were visualized using ECLTM western blotting detection reagents (GE Healthcare, Buckinghomshire, UK) with the signals detected by the Image Station 4000R (Kodak, New Haven, CT, USA).

SDS-PAGE and in-gel digestion

One hundred and fifty micrograms of proteins from the supernatant were separated by a 5-well Novex® 4–20% Tris–glycine gel (Invitrogen, Carlsbad, CA, USA; Fig. 1). The gel was then fixed in 40% methanol/10% acetic acid for 15 min, stained with colloidal Coomassie Blue solution (Invitrogen, Carlsbad, CA, USA) for 45 min, and destained in 40% methanol/10% acetic acid for 24 h. The bands were excised and minced into \(1 \times 1 \) mm of smaller pieces, sufficiently destained with 50% acetonitrile/25 mM ammonium bicarbonate, dehydrated with 100% acetonitrile, and dried in a vacuum centrifuge.

A standard trypsin digestion procedure was performed. In brief, the gel pieces were reduced in 20 mM dithiothreitol for 45 min at 55°C, carboxyamidomethylated in 55 mM iodoacetamide, and digested with trypsin (Promega, Madison, WI, USA) solution (10 ng/μl dissolved in 25 mM ammonium bicarbonate, pH 8.0) overnight at 37°C. The peptides were extracted twice with 0.1% trifluoroacetic acid and 0.1% trifluoroacetic acid/50% acetonitrile, respectively, and dried in a vacuum centrifuge. The volumes of the extraction were adjusted to 15 μl with 0.1% trifluoroacetic acid, of which 5 μl was loaded in LTQ-Orbitrap for LC-MS/MS analysis.
LC-MS/MS analysis

For LC-MS/MS analysis, each digestion product was separated by a 60-min gradient elution with the Dionex capillary/nano-HPLC system (Dionex, Sunnyvale, CA, USA) at a flow rate of 0.250 μl/min that is directly interfaced with the Thermo-Fisher LTQ-Orbitrap mass spectrometer (Thermo Fisher, San Jose, CA, USA) operated in a data-dependent scan mode. The analytical column was a fused silica capillary column (75 μm ID, 100 mm length, Upchurch, Oak Harbor, WA, USA) packed with C-18 resin (300 A, 5 μm, Varian, Palo Alto, CA, USA). Mobile phase A was consisted of 0.1% formic acid, and mobile phase B was consisted of 100% acetonitrile and 0.1% formic acid. The 60-min gradients with 250 nl/min flow rate for B solvent went from 0% to 55% in 34 min and then in 4 min to 80%. The B solvent stayed at 80% for another 8 min and then decreased to 5% in 8 min. Another 6 min was used for equilibration, loading, and washing. The LTQ-Orbitrap mass spectrometer was operated in the data-dependent acquisition mode using the Xcalibur 2.0.7 software. The experiment consisted of a single full-scan mass spectrum in the Orbitrap (400–1,800 m/z, 30,000 resolutions), followed by six data-dependent MS/MS scans in the ion trap at 35% normalized collision energy. The dynamic exclusion parameters were as follows: repeat count=1, repeat duration=30, exclusion list=100, and exclusion time=90.

Data analysis

**Bioinformatics analysis of proteins**

The MS/MS peak lists were searched against the IPI mouse database using BIOWORKS software (version 3.3.1 SP1, Thermo Fisher Scientific Inc.) with the following parameters: monoisotopic masses of 20 ppm on MS, fully tryptic specificity, one missed cleavage sites allowed, cysteine carbamidomethylation as a fixed modification, and 1 Da on MS/MS and oxidation of methionine as the variable modifications PepQuan from BIOWORKS was used to
extract the quantitative ratios of heavy peptides to light peptides. The peptides data were further filtered by selecting proteins with at least two unique peptides and the perspective value ($E$ value)<10E-3. The ratios of heavy peptides to light peptides were further confirmed by checking the individual MS peaks of the peptides using software Xcalibur (version 2.0.7, Thermo Fisher Scientific Inc.; Fig. 1). The proteins data were also globally analyzed using an online analysis tool, Protein Interrogation of Gene Ontology and KEGG databases (PIGOK, http://pc4-133.ludwig.ucl.ac.uk/pigoksum.html), by submitting IPI access number of all identified proteins [11]. In addition, the proteins that related to cell growth and differentiation were further clustered and analyzed according to the existing protein data and literature. Protein symbols and their full names were listed in Table 1.

Statistics

Data are expressed as mean ± SEM. Student's $t$ tests were used to determine differences between the pairs of DEX and CON groups. Analysis of variance was used to compare the differences among values of different culture days in DEX or CON group. Post hoc analyses were performed with Newman–Keuls tests. Differences were regarded as significant if $P<0.05$.

Results

Effects of DEX on ALP activity, proliferation, and apoptosis of MC3T3-E1 cells

When grown in a normal medium, the ALP activities of MC3T3-E1 cells did not considerably change up to 10 days. However, while the MC3T3-E1 cells were cultured in a normal medium containing $10^{-6}$ M DEX, the ALP activities of the cells decreased significantly on days 4, 7, and 10 compared with that on day 1 ($P<0.01$; Fig. 2a). The cell proliferation assay demonstrated that $10^{-6}$ M DEX significantly reduced the value of OD 590 in MC3T3-E1 cells when compared with control ($P<0.001$) on day 7 (Fig. 2b). In apoptosis assay, $10^{-6}$ M DEX significantly increased the apoptosis of cells on days 4, 7, and 10 compared with control (Fig. 2c). These data indicate that DEX inhibited MC3T3-E1 cell proliferation and differentiation and induced its apoptosis.

Proteomic profiles of SILAC proteins

A total of 1,186 SILAC proteins with $E$ value <1.0E-03 and at least two unique peptides (at least one peptide labeled by lysine) were identified by LC-MS/MS. The natural logarithm of the proteins' DEX/CON SILAC ratios was shown in Fig. 3a. The histogram has one main peak at zero, indicating that a major proportion of proteins have the SILAC ratios of 1. The shoulder peaks were in equilibrium between −0.5 and 0.5, but the shoulder peak between 0.5 and 0.9 was significantly higher than the peaks between −0.5 and −0.9 (Fig. 3a).

The data were further analyzed using PIGOK as described previously. First, in the categorization of cellular components of the identified proteins, the most matched proteins are membrane, nucleus, and cytoplasm proteins. Secondly, the pathways that are related to the osteoblast differentiation, such as MAPK-signaling pathway, calcium-signaling pathway, and insulin-signaling pathway, were among the most matched KEGG-signaling pathways (Fig. 3b). Finally, in biological process aspect, the most matched proteins were classified into multicellular organismal development, cell differentiation, and response to stimulus (Fig. 3c).

DEX stimulus affected the levels of “housekeeping proteins”

The mean SILAC ratios of certain “housekeeping proteins” such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Fig. 4a), actin, cytoplasmic 1 (ACTB), and actin, alpha skeletal muscle (ACTA) were closely to 1. Interestingly, the mean ratios of tubulin alpha-1 chain (TUBA1A; Fig. 4b), tubulin beta-2B chain (TUBB2B), and tubulin beta-5 chain (TUBB5) were significantly increased to 4.82, 3.94, and 3.39, respectively (Table 1).

DEX inhibited ATP synthase and transitional endoplasmic reticulum ATPase

We found that DEX treatment reduced the protein levels of some ATP synthase, such as ATP synthase O subunit, mitochondrial precursor (ATP5O), ATP synthase D chain, mitochondrial (ATP5H), ATP synthase subunit alpha, mitochondrial precursor (ATP5A1), and ATP synthase B chain, mitochondrial precursor (ATP5F1; Fig. 4c). The expression of ATP5O was also confirmed by Western blot (Fig. 5).

DEX upregulated S100A11, S100A6, S100A4, and S100A10 proteins

S100 proteins are involved in a variety of cellular processes, such as cell growth and cell differentiation [12]. In the present study, we found that the expression of protein S100A11 (Fig. 4d), S100A6, S100A4, and S100A10 was increased in the DEX group with the mean SILAC ratio of 4.05, 1.83, 1.62, and 1.52, respectively. The
## Table 1 Quantitative analysis of proteins treated with 10^{-6} M DEX in MC3T3-E1 cells

<table>
<thead>
<tr>
<th>IPI number</th>
<th>Protein names</th>
<th>Gene names</th>
<th>$E$ value</th>
<th>Pept. Ratio (T/C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPI00135284</td>
<td>Similar to glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Gapdh</td>
<td>1.00E-30</td>
<td>9</td>
</tr>
<tr>
<td>IPI00110850</td>
<td>Actin, cytoplasmic 1</td>
<td>Actb</td>
<td>1.00E-30</td>
<td>12</td>
</tr>
<tr>
<td>IPI00110827</td>
<td>Actin, alpha skeletal muscle</td>
<td>Acta</td>
<td>1.44E-12</td>
<td>9</td>
</tr>
<tr>
<td>IPI00110753</td>
<td>Tubulin alpha-1 chain</td>
<td>Tub1a</td>
<td>2.58E-11</td>
<td>5</td>
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<tr>
<td>IPI00109061</td>
<td>Tubulin beta-2B chain</td>
<td>Tubb2b</td>
<td>1.67E-15</td>
<td>1</td>
</tr>
<tr>
<td>IPI00117352</td>
<td>Tubulin beta-5 chain</td>
<td>Tubb5</td>
<td>2.50E-08</td>
<td>1</td>
</tr>
</tbody>
</table>

### ATP synthase and transitional endoplasmic reticulum ATPase

| IPI00118986 | ATP synthase O subunit, mitochondrial precursor | Atp5o | 1.46E-09 | 2 | 0.21±0.06 |
| IPI00230507 | ATP synthase D chain, mitochondrial | Atp5h | 7.88E-14 | 4 | 0.37±0.08 |
| IPI00130280 | ATP synthase subunit alpha, mitochondrial precursor | Atp5a1 | 2.50E-08 | 1 | 3.39^b |
| IPI00341282 | ATP synthase B chain, mitochondrial precursor | Atp5f1 | 1.09E-10 | 3 | 0.52±0.11 |
| IPI00622235 | Transitional endoplasmic reticulum ATPase | Vcp | 1.04E-11 | 4 | 0.42±0.09 |

### S100 proteins

| IPI00119202 | Protein S100A11 | S100a11 | 2.17E-11 | 4 | 4.05±1.65 |
| IPI00121427 | Protein S100A6 | S100a6 | 1.09E-10 | 3 | 1.83±0.08 |
| IPI00124096 | Protein S100A4 | S100a4 | 1.72E-12 | 3 | 1.62±0.15 |
| IPI00222555 | Protein S100A10 | S100a10 | 3.13E-08 | 2 | 1.52±0.21 |

### Small Ras GTPase and Ras-related proteins

| IPI00130095 | Ras GTPase-activating protein-binding protein 1 | G3bp1 | 4.79E-05 | 3 | 0.59±0.04 |
| IPI00467447 | Ras GTPase-activating-like protein IQGAP1 | Igqap1 | 7.77E-15 | 5 | 1.52±0.26 |
| IPI00114560 | Ras-related protein Rab-1A | Rab1A | 1.12E-06 | 3 | 0.61±0.04 |
| IPI00137227 | Ras-related protein Rab-2A | Rab2A | 4.88E-09 | 3 | 0.6±0.14 |
| IPI00408892 | Ras-related protein Rab-7 | Rab7 | 3.71E-10 | 2 | 0.55±0.05 |

### Myosins

| IPI00123181 | Myosin-9 | Myh9 | 2.28E-09 | 10 | 3.31±2.04 |
| IPI00114894 | Isoform 1 of myosin-11 | Myh11 | 9.95E-10 | 3 | 2.09±0.44 |
| IPI00109044 | Myosin light chain, regulatory B-like | Myl^a | 1.09E-11 | 2 | 4.77±1.32 |
| IPI00264053 | Similar to myosin light polypeptide 6 | Myl^a | 1.06E-09 | 3 | 10.45±4.9 |

### Apoptosis and stress proteins

| IPI00229080 | Heat shock protein 84b | Hsp84b | 6.51E-05 | 2 | 3.77±1.21 |
| IPI00308885 | 60 kDa heat shock protein, mitochondrial precursor | Hsp60 | 1.00E-30 | 8 | 1.57±0.13 |
| IPI00323483 | Programmed cell death 6-interacting protein | Pdcd6ip | 2.13E-06 | 1 | 1.53^b |
| IPI00331556 | Heat shock 70 kDa protein 4 | Hspa4 | 1.15E-08 | 2 | 0.91±0.15 |
| IPI00120684 | Apoptosis regulator BAX, membrane isoform alpha | BAX | 1.07E-05 | 3 | 3.98±0.28 |
| IPI00129526 | Heat shock protein HSP 90-alpha | Hsp90aa1 | 4.44E-15 | 4 | 0.95±0.19 |

### Others

| IPI00331361 | Myb-binding protein 1A | Mybbp1a | 9.89E-11 | 6 | 0.44±0.19 |
| IPI00116498 | 14-3-3 protein zeta/delta | Ywhaz | 2.03E-04 | 2 | 3.17±0.42 |
| IPI00273803 | 60 S ribosomal protein L15 | Rpl15 | 4.21E-04 | 3 | 2.23±0.72 |
| IPI00553798 | AHNAK nucleoprotein isoform 1 | Ahnak | 8.33E-08 | 3 | 2.56±0.42 |
| IPI00319509 | Aminopeptidase N | Anpep | 1.45E-04 | 3 | 0.45±0.11 |
| IPI00230395 | Annexin A1 | Anxa1 | 2.28E-10 | 5 | 0.27±0.05 |
| IPI00132756 | Annexin A8 | Anxa8 | 9.98E-07 | 3 | 0.29±0.09 |
| IPI00315437 | BET1-like protein | Bet1l | 3.74E-05 | 2 | 7.08±2.93 |
| IPI00169916 | Clathrin heavy chain | Clc | 3.45E-09 | 4 | 2.00±0.33 |
| IPI00395038 | Exportin-1 | Xpo1 | 3.47E-06 | 3 | 7.90±2.57 |
| IPI00380394 | Integrator complex subunit 3 | Ints3 | 9.92E-04 | 3 | 3.65±0.87 |
protein expression of S100A4 was further confirmed by Western blot analysis (Fig. 5).

Regulation of small Ras GTPase and Ras-related proteins

Interestingly, we found the SILAC ratio of Ras GTPase-activating-like protein 1 (IQGAP1) was increased to 1.52±0.26 (Table 1). However, the Ras GTPase-activating protein-binding protein 1 (G3BP1) demonstrated a decreased ratio of 0.59±0.04 (Table 1). In proteomic analysis of Ras-related proteins, the protein levels of Ras-related protein Rab-1A (RAB1A; Fig. 4e), Ras-related protein Rab-2A (RAB2A), and Ras-related protein Rab-7 (RAB7) were downregulated by $10^{-6}$ M DEX (Table 1), and the expression of RAB7 was confirmed by Western blot (Fig. 5).

Regulation of myosins and other proteins

In the present study, we detected the drastic upregulation of some myosin proteins such as myosin 9 (MYH9; Fig. 4f) and isoform 1 of myosin 11 (MYH11; Table 1). Additionally, we found myosin light chain, regulatory B-like, and protein similar to myosin light polypeptide 6 (Fig. 4g) that had SILAC ratios as high as 4.77±1.32 and 10.45±4.9, respectively (Table 1). We also found downregulation of myb-binding protein 1A (MYBBP1A; Fig. 4h), annexin A1 (ANXA1), and annexin A8 (ANXA8; Table 1). However, the levels of some other proteins, such as polymerase I and transcript release factor (PRF), peptidylprolyl isomerase B (PPIB), and integrator complex subunit 3 (INTS3), were up-regulated during the DEX treatment (Table 1).

Regulation of apoptosis and stress proteins

Our study also showed the overexpression of apoptosis regulator BAX (Fig. 4i), heat shock protein 84b (HSP84B), 60 kDa heat shock protein (HSP60), and programmed cell death 6-interacting protein (PDCD6IP). However, the expression of heat shock 70 kDa protein 4 (HSPA4) and heat shock protein HSP 90-alpha (HSP90AA1) remains stable (Table 1).

Discussion

In accordance with the previous data [13–16], we demonstrated that a high dose of DEX inhibited osteoblast differentiation and cell proliferation but promoted apoptosis in MC3T3-E1 cells on day 7. Using SILAC-based quantitative proteomics, we identified the drastic protein changes in tubulins, IQGAP1, S100 proteins, myosin proteins, ATP synthase, G3BP-1, Ras-related proteins, and apoptosis and stress proteins in the present study. Firstly, DEX did not affect the expression of GAPDH and actins but significantly increased the expression of TUBA1A, TUBB2B, and TUBB5. The alpha and beta tubulins represent two major components of microtubules. Heterozygous mutations in TUBB2B cause malformations of the brain, manifesting as asymmetrical polymicrogyria
Mutations in the TUBA1A were reported in patients with lissencephaly [18], and some affected patients show bone dysplasia [19]. TUBB5 have been proven to be upregulated by Hoxc8 overexpression, and the interaction between Hoxc8 and Smad1 is the major initiatory mechanism of osteoblast differentiation in BMP signaling [20, 21]. Therefore, DEX may inhibit cell proliferation through the reduction of ATP synthases. The VCP is necessary in the export of misfolded proteins from the endoplasmic reticulum to the cytoplasm, where they are degraded by the proteasome [23, 24]. The impaired VCP level in this study may lead to the accumulation of misfolded proteins and, in addition, affect the cell proliferation and osteoblast differentiation.

Interestingly, though their biological functions in osteoblast differentiation are poorly understood, some members of S100 proteins were upregulated by DEX treatment. The S100 proteins are multifunctional signaling proteins, involving in the regulation of diverse cellular processes such as contraction, motility, cell growth, differentiation, cell cycle progression, transcription, and secretion [12]. The S100 proteins also show remarkably cell- and tissue-specific expression patterns [12]. For example, S100A11 may inhibit or stimulate cell growth in human keratinocytes under different circumstances [25], and S100A6 is an intracellular protein that is overexpressed in human osteosarcoma [26–29]. S100A10 is involved in the intracellular trafficking of a set of plasma membrane ion channels and receptors [30, 31], and DEX has been proven to upregulate S100A10 expression in two human epithelial cell lines [32]. Additionally, S100A4 is a negative regulator of mineralization that declines before the onset of mineralization in human mesenchymal stem cells [33]. In this study, the increased S100A4 expression on day 7 after DEX treatment may be caused by impaired osteoblast differentiation, and we demonstrated that DEX may inhibit cell proliferation and osteoblast differentiation through upregulation of S100A11, S100A6, S100A4, and S100A10 proteins in MC3T3-E1 cells. Overall, regulation of S100 proteins may be part of the compensatory process that appears in DEX-treated cells. However, the functions of different S100 proteins on osteoblast differentiation are distinct and require further investigation.

The next is the Ras superfamily of small GTPases, which regulates gene transcription, cell growth, and cellular transformation. DEX has been shown to regulate some members of the Ras superfamily of small GTPases [34, 35]. The Ras GTPase-activating protein (GAP) inactivates small GTPases by cycling them from an active GTP bound state to an inactive GDP bound state. IQGAP1 is a homology to GAPs but appears to inhibit the GTPase activity [36, 37]. In this study, DEX increased the protein level of IQGAP1, indicating that DEX may decrease the GTPase activity through IQGAP1. The protein level of G3BP-1 pertains to Ras signaling, NF-κB signaling, the ubiquitin proteosome pathway, and RNA processing [38–41]. In this study, the protein level of G3BP1 was inhibited by 10^{-6} M DEX, synthases synthesize ATP from ADP and inorganic phosphate and embody two of the major cellular energy transduction mechanisms [22]. Therefore, DEX may inhibit cell proliferation through the reduction of ATP synthases. The VCP is necessary in the export of misfolded proteins from the endoplasmic reticulum to the cytoplasm, where they are degraded by the proteasome [23, 24]. The impaired VCP level in this study may lead to the accumulation of misfolded proteins and, in addition, affect the cell proliferation and osteoblast differentiation.

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indicating that DEX may affect Ras signaling pathway through regulation of G3BP1. The Rab proteins regulate the membrane vesicular trafficking pathways [42]. Rab1A is one isoform of Rab1 that is located in the endoplasmic reticulum, pre-Golgi intermediates, and the Golgi stack, suggesting that it regulates anterograde transport of cargo between the endoplasmic reticulum and the Golgi apparatus [43]. Rab2, on the other hand, regulates the anterograde transport from the endoplasmic reticulum to the Golgi apparatus [44]. Rab7 participates in the endosomal pathway [45–48]. In this regard, DEX may inhibit cell proliferation and osteoblast differentiation through the reduction of membrane traffic process.

The reason why some myosins are upregulated remains unclear. The myosin light polypeptide 6 is a regulatory light chain of myosin and expressed also in the fibroblasts and some tumor tissues or cells. MYH9 is the nonmuscle myosin heavy chain and has been shown to be temporarily suppressed during osteoclastogenesis [49]. Myosin-11 is a smooth muscle cell differentiation marker and is upregulated during myogenic differentiation in smooth muscle cells or mesenchymal stem cells [50, 51]. The myosin light chain regulatory B-like is similar to myosin regulatory light chain 2, atrial isoform (MY17), which is a calcium-binding chain.

Finally, we identified that a pro-apoptotic protein, BAX, increased 3.98 times during the DEX treatment. BAX has been shown to promote apoptosis through formation of mitochondrial outer permeabilization pore [52], while Bcl-2 inhibits its formation. We also identified overexpressed PDCD6IP (Aip1 or Alix). PDCD6IP plays a role in modulating apoptosis as it interacts with apoptosis-related proteins such as programmed cell death protein 6 (PDCD6)
and endophilins [53, 54]. There are quantitative modification of some stress proteins, such as heat shock protein 84b (Hsp84b) and 60 kDa heat shock protein (Hsp60), but their functions in apoptosis remain unclear.

In conclusion, $10^{-6}$ M DEX inhibited the osteoblast differentiation and proliferation but promoted apoptosis in MC3T3-E1 cells on day 7. The upregulated levels of tubulin (TUBA1A, TUBB2B, and TUBB5), IQGAP1, S100 proteins (S100A11, S100A6, S100A4, and S100A10), myosin proteins (MYH9 and MYH11), apoptosis and stress-related proteins (BAX, Hsp84b, Hsp60,

Fig. 4 Mass spectra of peptides from identified representative proteins (a–i). Filled circle: main peak for SILAC ratio calculation in DEX group, light lysine labeled (L); filled triangle: main peak for SILAC ratio calculation in CON group, heavy lysine labeled (H).

Fig. 5 Western blot analysis of protein ATP5O, Rab7, and S100A4 with β-actin as internal control.
PDCD6IP), and the downregulated protein levels of ATP synthase (ATP5O, ATP5H, ATP5A1, and ATP5F1), G3BP-1, and Ras-related proteins (Rab-1A, Rab-2A, and Rab-7) may be critical in the mechanism of the DEX-induced results. Such protein expression changes may be of pathological significance in coping with GCOP.

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Conflicts of interest None.

References


